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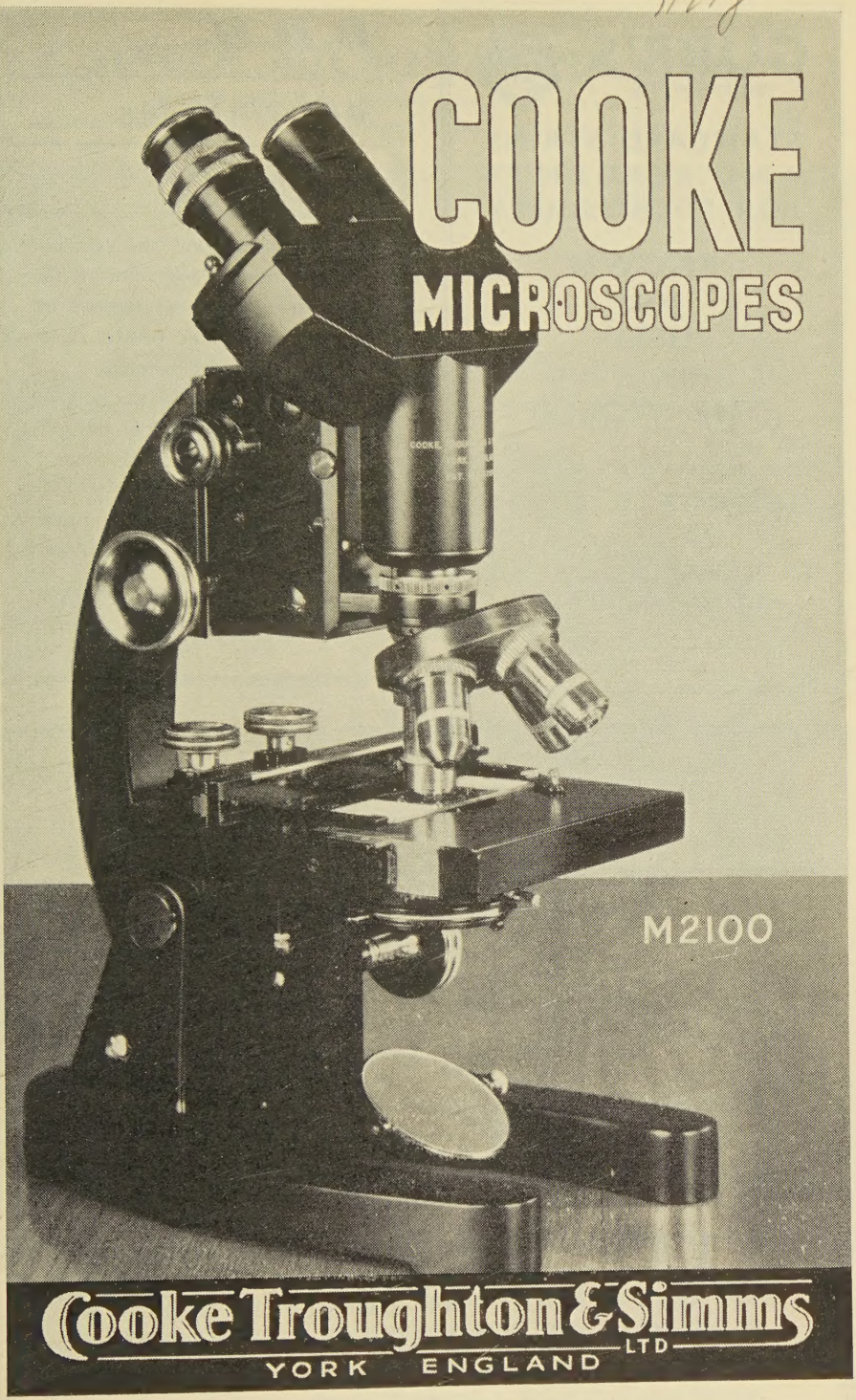

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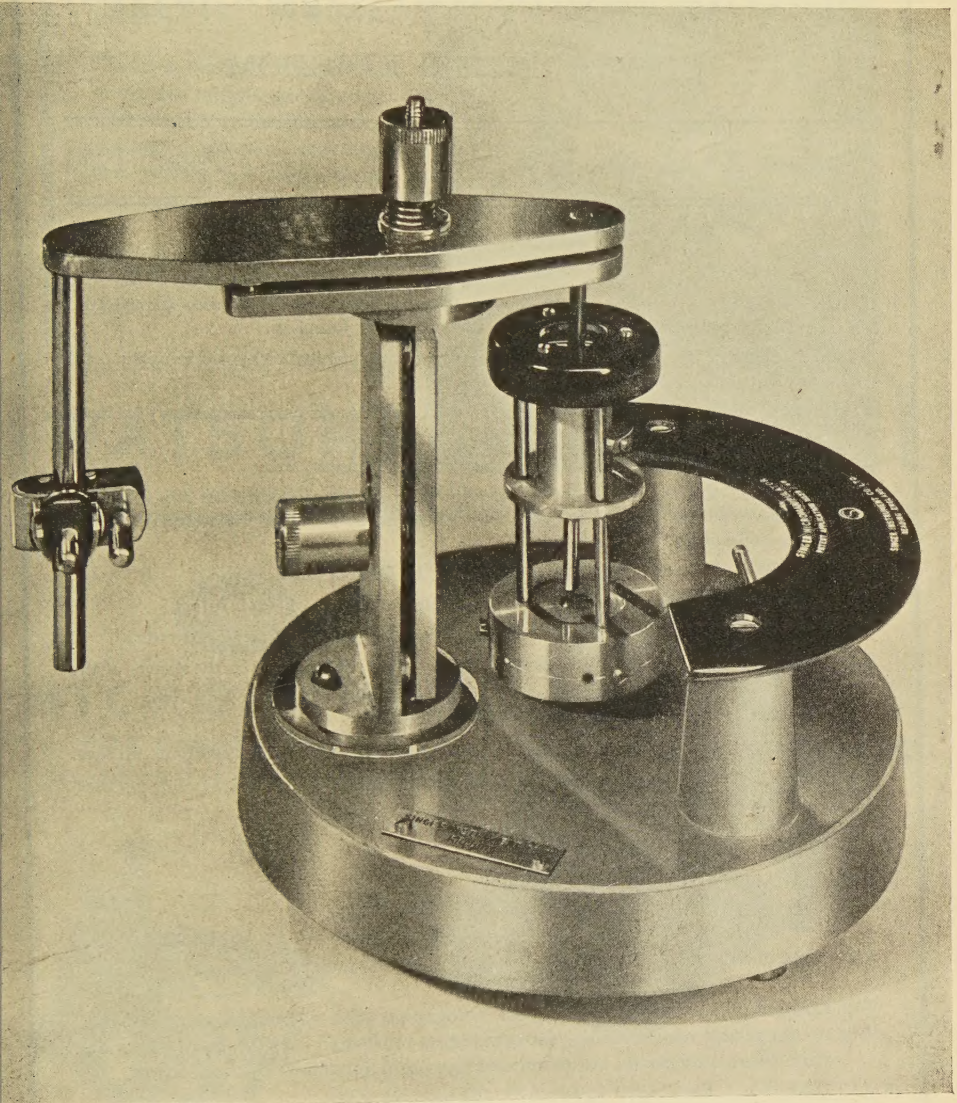

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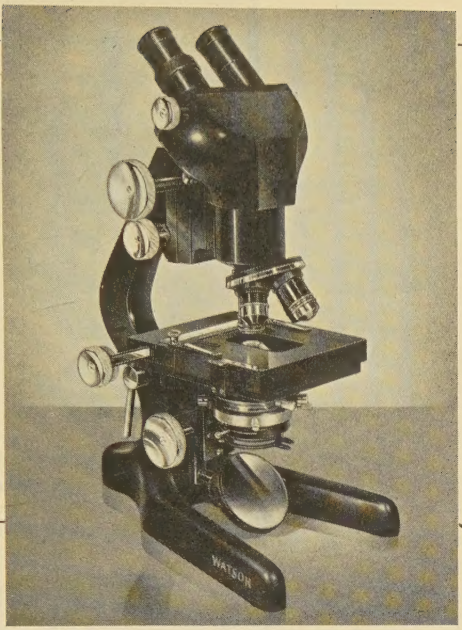

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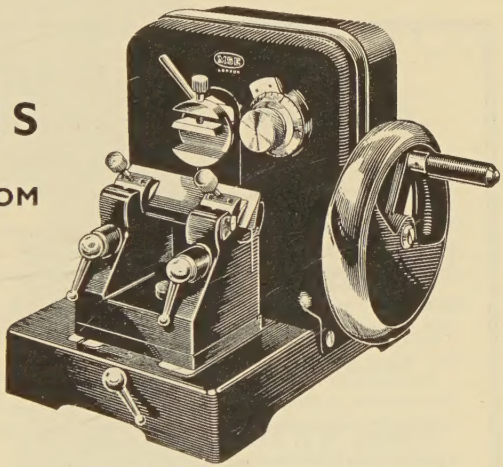

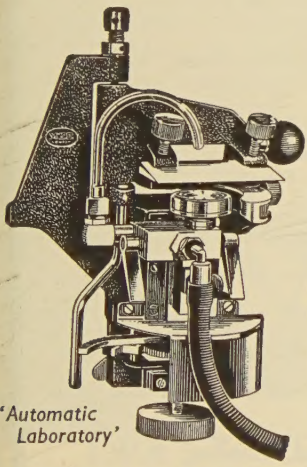

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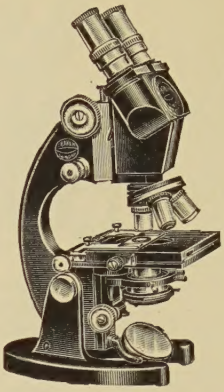

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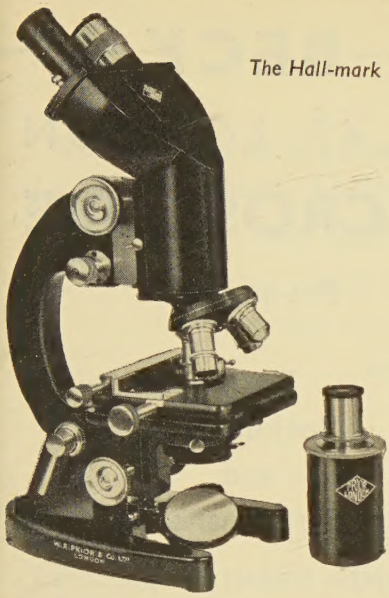

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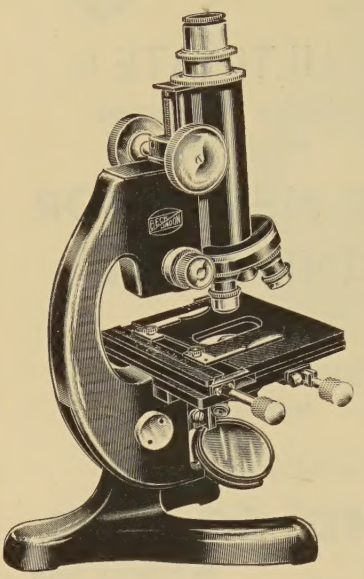

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The Digestive Mechanism of the European Squids *Loligo vulgaris, Loligo forbesii, Alloteuthis media,* and *Alloteuthis subulata*

By ANNA M. BIDDER

(*From the Department of Zoology, Cambridge University*)

With two Plates

Contents

Summary

1. The digestive mechanism has been investigated in *Loligo forbesii*, *L. vulgaris*, *Alloteuthis media*, and *A. subulata*. Histology and morphology were examined on living and fixed material and the feeding of *L. vulgaris* was observed in the living animal.

2. The food is bitten by the jaws and rapidly swallowed; the radula has no rasping action.

3. Preliminary digestion takes place in the stomach, which is cuticle-lined.

4. The caecum is a complex organ, containing the opening of the mid-gut gland. This opening is connected with the closing mechanism of the caeco-intestinal opening in such a way that the hepato-pancreatic secretion can be directed either to the caecum or to the stomach. The anterior part of the caecum contains a ciliary collecting mechanism whose main groove leads along the intestine towards the anus.

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5. The posterior part of the caecum is a simple ciliated sac where digestion is completed, food absorbed, and pancreatic secretion stored between meals. Solid food other than particulate is never found in the caecum.

6. The intestine is a short, straight tube, lined with a ciliated and mucous epithelium. Absorption also takes place here, and continues after the caecum has ceased absorbing.

7. The junction of intestine and rectum is considered to be defined by the replacement of the ciliated epithelium by one with a hyaline border.

8. The rectum is short; in the region of the rectal sphincter it is lined with cells bearing curious retractile processes, whose possible function is discussed.

9. The mid-gut gland secretes all digestive enzymes; it is divided into two unequal and markedly different glands: the so-called 'liver' and 'pancreas'. These are connected in series, so that hepatic secretion flows through the main lumen of the pancreas to reach the caecum.

10. Hepatic secretion is passed into the caecum only during digestion. The liver has a single type of cell in which food reserves also accumulate. The hepatic duct can be closed by a sphincter.

11. Pancreatic secretion accumulates in the caecum between meals and apparently passes to the stomach during digestion. The structure and rhythm of the cells of the pancreas and the nature of its blood-supply suggest some second activity, possibly directed towards the blood-stream, alternating with that of enzyme-secretion.

12. Food is not absorbed in the mid-gut gland.

13. The structural and functional peculiarities of the digestive system of these squids may be related to an exceptionally speedy and efficient digestive mechanism, well fitted to the life of a perpetually swimming, active predator.

Introduction

THE anatomy and physiology of the Cephalopoda present a fascinating problem to the student of functional morphology.

It is classic to correlate their active predatory life and highly developed organs of locomotion with eye and brain, heart and blood-system, prehensile tentacles and powerful beak; and it has long been known that prey of astonishing size and activity can be captured and killed by these means. The problem of how and where the food so obtained is digested and absorbed, of the extent to which the cephalopod digestive system resembles and differs from that of other Mollusca is clearly one of considerable interest.

The preliminary study described in these pages was made on the two European species of the genus *Loligo*, *L. forbesii* Steenstrup 1856 and *L. vulgaris* Lam. 1799, with supplementary observations on *Alloteuthis subulata* (L. 1798) Naef 1921 and *A. media* (L. 1758) Naef 1921: no significant difference was detected between the European species of these two genera. These forms were originally selected by the present writer because a study of the larval function of the gut during the period of yolk-absorption (Portmann and Bidder, 1928) drew attention to the whole question of the digestive mechanism in the adult. The material proved to be ideal in that the great transparency of the tissues makes it possible to observe much of the digestive mechanism in the living, feeding animal as it swam in a tank. Against this rare advantage must be set the great disadvantage that these animals are so delicate and highly strung that they seldom survive capture unless very special precautions are

observed, and then will often die in captivity before they have recovered sufficiently to feed. As a result, much of the histological material was not derived from animals at a known stage of digestion, beyond the very crude measure given by an examination of the stomach contents, while the observations on the ciliary currents of the gut were largely made on moribund material.

Nevertheless it has proved possible to reconstruct a digestive mechanism which proved to have so many interesting features as to justify the publication of its description, despite the many gaps which, it is hoped, may one day be filled.

The actual observations on living material on which this account is based were largely made in the years 1929–31, with a few additional observations in the summer of 1939 and spring of 1947.

Literature

Very little published work exists on the gut of *Loligo* and none on that of *Alloteuthis*. Short accounts of the morphology are given in Cuvier's monograph on the Mollusca (1817), in the *Leçons d'anatomie comparée* (1805, 1837–40), and in Bourquelot's second paper on the digestive enzymes of the Cephalopoda (1885), and a full account of the digestive system is contained in Williams's monograph (1909) on *Loligo pealeii* Les., and can for the most part be applied to the European species. This monograph did not reach my notice until most of my work on *Loligo* was completed. Through the kindness of Miss Grace Pickford I was able to examine some specimens of *L. pealeii* and confirm that the major differences between my account and that of Williams correspond, in fact, to specific differences. The action of the jaws is discussed by Jordan (1913, p. 353) and the jaws and radula are figured by Naef (1923), for *L. vulgaris* and for *L. forbesii*. (Heinrich's (1904) account of the jaws of '*Loligo todarus*' refers in fact to *Ommatostrephes sagittatus* (Lam.) d'Orbigny.) The histology of the alimentary canal is described shortly by Williams (1909), of the liver briefly by Cuénot (1907), and of the pancreas by Vigelius (1881, 1883). The histology of the alimentary canal is described in detail by Gariaeff (1915) in a long comparative paper on the histology of the Cephalopod gut. This paper is entirely in Russian; through the kindness of Dr. B. P. Uvarov I was able to obtain a translation of the greater part. The digestive enzymes are discussed very briefly by Bourquelot (1882, 1885) and mentioned by Williams (1909); both these authors and Gariaeff (1915) discuss the function of the various parts of the gut; Sellier (1910) gives an account of the proteolytic enzymes. Romijn (1935) investigated the carbohydrate-splitting enzymes. The part played by the larval liver and pancreas in the absorption of the yolk in both *Loligo* and *Alloteuthis* is discussed by Portmann and Bidder (1928). Most of the best known studies of Cephalopod digestive mechanisms either do not mention *Loligo*, or do it so passingly that it is impossible to refer any statement from them to this genus.

Material

Loligo forbesii (Steenstrup 1856) and *Alloteuthis subulata* (L. 1758) Naef 1921 were obtained at Plymouth, *L. vulgaris* Lamark 1799 and *A. media* (L. 1758) Naef 1921 at Naples. No difference in structure or histology has been detected between any of these species, which are therefore treated together. The Plymouth species were chiefly used for morphology and the ciliary currents, which were worked out almost entirely on *Loligo*; some histological material was also obtained from both species, and compared with that obtained at Naples. The living Naples material was all *L. vulgaris*. This species appears to be slightly less sensitive than *L. forbesii*, and it was possible to keep half-grown specimens (less delicate than sexually mature animals) in captivity, and carry out the feeding experiments described on p. 5 and the observations described on pp. 9 ff. In addition, a certain number of young forms (mantle-length 15–50 mm.) were obtained, many of them alive, in which the movements of the digestive organs could be watched under the binocular dissecting microscope. Some of these were probably specimens of *Alloteuthis*, as the two genera are difficult to distinguish when young; but the observations were consistent, so that this possibility only shows the close similarity between the digestive mechanisms of all four species.

This agreement is all the more remarkable as the common American species of *Loligo*, *L. pealeii*, differs from all four European species in a significant point of gut structure (see p. 20).

Technique

The *morphology* was studied in fresh and preserved material of two distinct types: that which was received alive and decapitated for examination, and that which had died in the trawl, or after capture, apparently from 'shock', without visible injury. In material of the first type the tissues remain alive and transparent for a considerable time after death, and the gut shows active and even violent peristaltic contractions, while dissection or manipulation of any kind is liable to start local tonic contractions, producing conditions which are never found in freshly killed material, and are therefore to be regarded as post-mortem effects. In material of the second type the body becomes quickly rigid and opaque, and it was rare to find any but the faintest traces of peristaltic activity in the gut, but post-mortem contraction is of frequent occurrence.

For general morphology material was fixed and preserved in 5 per cent. formalin (2 per cent. formaldehyde), neutralized, and usually made up in sea-water; this was found to preserve the organs with very little shrinkage or loss of colour and the slight maceration did not interfere with the study of general structure, which was confirmed on fresh material. For preservation the mantle was always opened ventrally, so that only the thin body-wall covered the digestive organs.

Serial sections of complete small digestive systems, and plasticine models on an enlarged scale were used in interpreting the details of structure.

The *feeding experiments* were carried out on *Loligo* by the use of traceable foods, originally in the hope of tracing absorption. *Loligo* is difficult to maintain in captivity but living *Loligo* were obtained at Naples, where fishing by man-hauled nets from small boats made it possible to transfer the animals directly from the mouth of the net to a bucket of sea-water. Small shoals of immature animals about 70 mm. in mantle-length, taken in this way, will live for some weeks in the deep, cool tanks of the aquarium and will feed fairly readily. The tanks used for the feeding experiments, however, were, for practical reasons, smaller, shallower, and lighter than the aquarium tanks and the squid were not really healthy or at ease in them; they died more quickly than in the aquarium tanks, often refused to feed at all, and were clearly overstimulated. The capture of one member of a shoal threw the others into a state of agitation which apparently reduced the likelihood of their feeding at a later time.

Fragments of small fish were used as food, and treated with a mixture of iron saccharate and finely ground carmine, and with nile blue (sulphate). These substances were embedded in agar-agar jelly or gelatine jelly, and strips of jelly were inserted in the fish.

It was not easy to persuade the squid to feed, as the mere approach of an observer to the tank, and the dropping of food into the water, frightened them so much that the food had often fallen to the bottom of the tank before they had recovered. Once food had fallen on the bottom, it was rarely taken again. Pieces of food suspended on threads in mid-water were always ignored. The most successful method was to balance food on a loop of wire, fixed into the end of a long piece of glass tubing, so that neither operator nor tool was visible to the squid, and to let the food fall in as gently as possible. Chiefly by this means, between fifteen and twenty animals were fed, during a total period of 3 months.

Animals so fed with iron saccharate were decapitated immediately after capture and opened ventrally. Small fragments of the mid-gut gland were fixed in various fixatives and the whole animal then immediately fixed in ammonium sulphide with Bouin-Dubosq. The solution used was: ammonium sulphide 1 part, 90 per cent. alcohol 11 parts, absolute alcohol 3 parts, an equal volume of alcoholic Bouin being added just before use. This avoided the precipitate obtained with aqueous Bouin (Yonge, 1926, p. 710) or with 90 per cent. alcohol, without the addition of absolute alcohol. After 24 hours the fixative was changed, and the whole digestive system cut transversely into blocks, which were embedded after washing well in 95 per cent. alcohol. Sections were stained with carmalum and treated with 10 per cent. potassium ferrocyanide followed by 1 per cent. hydrochloric acid.

Considerably longer times were required than those given by Hirsch (1925, p. 4) for hydrochloric acid or Yonge (loc. cit.) for potassium ferrocyanide and hydrochloric acid. About 1 hour in potassium ferrocyanide and 10–20 minutes in the acid were necessary to bring out the blue reaction for the iron. It is not certain how much of the colour obtained was derived from the iron saccharate, as some colour was found in animals which had received no iron. This colour

probably represents iron normally present and revealed by the technique. Three types of colouring were obtained: a brilliant greenish-blue in the pen and iridescent sheath of the ink-sac, present at all stages; a diffuse, very pale blue, rapidly fading, and appearing in the blood-system 2–4 hours after feeding and in the muscle after a longer interval; finally, some granules in the liver cell showed the characteristic Prussian-blue reaction, both with and without administered iron. This was the only reaction of interest to the present discussion (see p. 33). Material for histology was obtained from *L. vulgaris*, as already stated, by taking small fragments from each of the animals used in the feeding experiments and therefore killed at known intervals after the taking of food. Material from *L. forbesii* and *A. subulata* was obtained from animals decapitated as they came out of the trawl. The number of minutes which had elapsed since death was noted for each individual fixation.

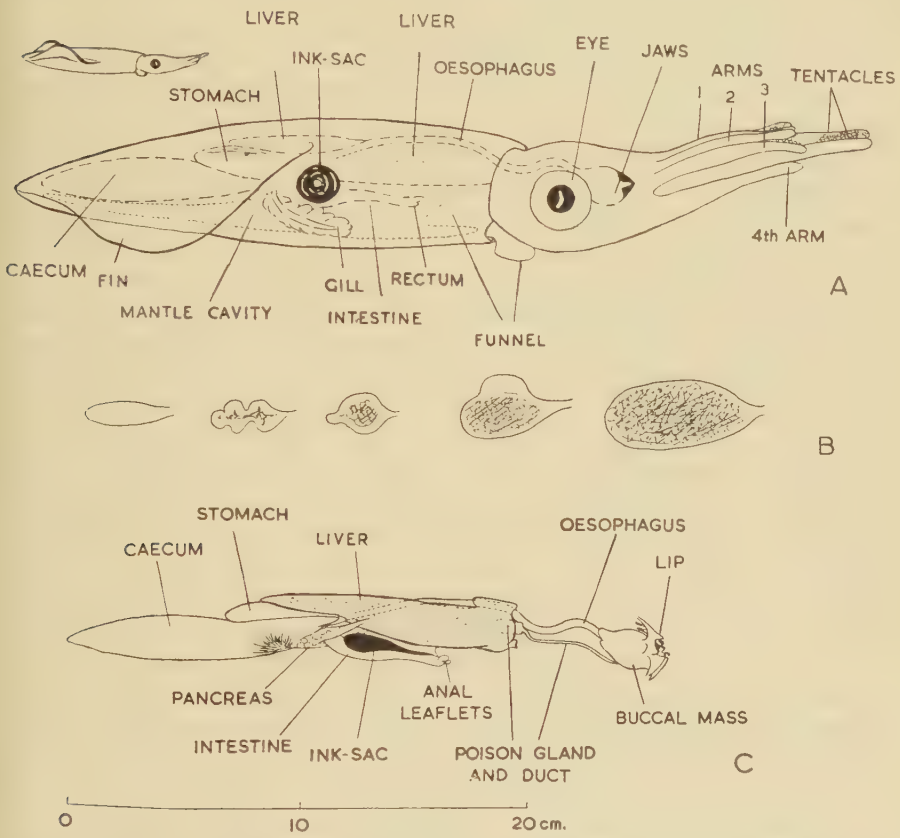
The material used was fixed in Zenker's fluid, Helly's Zenker-formol, Bouin-Dubosq with ammonium sulphide, 5 per cent. formalin, Flemming-without-acetic, and Champy. Sections were cut usually about 6μ thick, and stained with haemalum and eosin or orange G., Mallory, or (most generally useful) with iron haematoxylin (Geigy or Grübler), counter-stained with eosin and light green after Hollande's variation of Prenant's triple stain (Portmann and Bidder, 1928, p. 320) (the method is there attributed to Dubosq; the late Professor Dubosq later informed me that the method was due to Hollande). I have recently received from Professor Portmann the following modification: stain with iron haematoxylin; wash in running tap-water; distilled water; 0·25 per cent. aqueous eosin 2–6 hours (watch); rinse well with distilled water; 1 per cent. phosphomolybdic acid on the slide 10–15 minutes; rinse with tap-water; distilled water; alcohol series to 95 per cent. very quickly (differentiation of eosin); 0·5 per cent. light green in 95 per cent. (watch carefully); absolute alcohol on the slide; xylene; balsam. I have found that Mayer's haemalum can often profitably replace iron haematoxylin.

After Flemming or Champy, sections were sometimes stained with safranin and light green. Ethyl alcohol was used throughout the histology. The pictures obtained with the different fixatives agreed closely on many points, and could usually be equated to what had been seen in the living cell. The best results in general were obtained after fixation with Zenker or Helly. Bouin tended to produce shrinkage of the cytoplasm and a vesicular appearance in the nuclei.

Vital staining was principally confined to the liver and pancreas, but the mucous cells of the ciliated organ of *L. forbesii* were examined at Plymouth with methylene blue. A preliminary examination of the cells of the liver and pancreas was made at Naples with *L. vulgaris*. The living (and dying) cells were examined for general structure unstained, and with neutral red (Gurr), nile blue (sulphate), brilliant cresyl blue, and Janus green B (Höchst). Fresh tissues were tested for fatty substances with nile blue, Sudan III, and osmium tetroxide; *L. forbesii* and *A. subulata* were used at Plymouth, *L. vulgaris* at Naples and Banyuls-sur-Mer.

Observations on the Living Animal

Loligo and *Alloteuthis* are pelagic animals and *L. vulgaris* spends its life, when in captivity, swimming ceaselessly to and fro. The slender, torpedo-shaped body and wide, undulating fins, are shown in Text-fig. 1A. The



Text-fig. 1. A. Young living *Loligo vulgaris* as seen swimming in tank. Optical section of mantle-cavity in dotted outline. Pancreas and details of gills not visible in life. Oesophagus, stomach, and caecum only visible when containing food.

B. Stomach empty, half-full and actively churning, and fully distended.

C. Digestive system from animal's right side.

A, B, and C to same scale.

mantle-musculature is powerful and the animal, besides swimming gently to and fro with the fins, can swim very rapidly either backwards or forwards by ejecting water through the funnel. In swimming forwards the stream from the funnel is directed backwards, as described by Williams (1909, p. 43) for *L. pealeii* and by Russell and Steven (1930) for *Sepia*.

The main parts of the digestive system can be seen through the transparent body-wall and mantle: the jaws, the slender oesophagus and intestine, which

together form a U-shaped alimentary canal, the sac-like stomach and caecum, which open side by side into the bend of the U, and the liver, which occupies the anterior half of the body, as the caecum occupies the posterior half. The orientation used in this paper is that of the swimming animal: the pen is 'dorsal', the funnel and mantle-cavity 'ventral', the head and arms 'anterior', the pointed end of the body 'posterior'.

The only parts of the digestive system not visible in the living animal are the buccal glands and poison glands and the 'pancreas' (Text-fig. 2A, B), which is a spongy elaboration of the duct by which the liver opens into the caecum.

Loligo's natural food is fish, crustacea, and smaller squid, all of which have been found in the stomachs of captured animals. The cannibal habit of *L. pealeii* was observed in life by Verrill (1882, p. 354) and recorded from stomach-contents by Williams (1909, p. 2). Examination of the stomach-content of *Alloteuthis* showed a diet and method of feeding exactly as found for *Loligo*; i.e. the stomach contained bitten pieces of fish and crustacea. In the stomach of one female, of mantle-length 80 mm., the entire pen of another squid was found, measuring 19 mm. in length! The viscera had not been rejected as they would have been with a fish (see p. 9), and the stomach and ciliated organ of the prey were found nearly complete, together with the buccal mass, optic ganglia, &c. The body of a squid is soft compared with that of a fish or crustacean of the same size, yet it remains a mystery how such large fragments as those just described could pass through the brain and skull, by a relatively narrow passage, without inconveniencing the eater almost as seriously as the eaten.

In the work here described, only small dead fish and pieces of fish were used. A healthy captive squid will eat once or twice a day; when at liberty, the squid may feed more often, as shown by the stomach-contents of captured animals; but a large number of animals brought in have the stomach empty or containing only indigestible residue, so that (as would be expected with predators) the animals are not continuous feeders even in freedom.

Williams (1909, p. 43) states that *L. pealeii*, when capturing living fish, may lie in wait for them on the bottom of the tank, to which they anchor themselves by their tentacles, and Drew (1911, pp. 337–8) speaks of a squid lying on the bottom 'in the attitude habitually assumed by resting squid'. I have not seen *L. vulgaris* in pursuit of living fish, but, in my experience, it never lies on the bottom unless it is sick and within a few hours of death. There is a clearly marked difference in behaviour between the two species, and I was once informed by the late Dr. E. Sereni, who had worked both at Naples and Woods Hole, that *L. pealeii* is far hardier and more resistant to handling and operation than *L. vulgaris*. The differences may be correlated with their differences in distribution: *L. vulgaris* is usually to be found in 40–50 m. or in deeper waters, while *L. pealeii*, according to Verrill (1882) and Bigelow (1925), habitually occurs in shallow littoral waters. Thus to *L. pealeii* the bottom might be a familiar object, and contact with solid objects a familiar experience, while neither would ever enter into the life of *L. vulgaris*. This would account

for the difficulty shown by *L. vulgaris* in recognizing a piece of food when it has fallen on to the bottom, since, in this squid's normal life, an object which has fallen has gone for ever.

Once a piece of food has been taken by *L. vulgaris*, the procedure is always the same. The fish is seized behind the head and held obliquely with the tail uppermost and is so carried until the head is bitten off and dropped. This takes from 2 to 5 minutes. The trunk of the fish is then held horizontally in the arms, in line with the body of the squid, with the tail sticking out between the arms, and is carried round in this position until the end of the meal. For the first 5 minutes or so the *Loligo* swims backwards only, round and round the tank, often appearing nervous and excitable, with the chromatophores rapidly expanding and contracting in uneven patches. Afterwards it becomes quieter and, for the rest of the meal, swims gently to and fro with contracted chromatophores.

The movements of the jaws (Text-fig. 1A) during the meal are clearly visible. Usually they keep their normal vertical position in the head and work about a horizontal axis; sometimes, however, the buccal mass rotates about the oesophagus, until the jaws are almost horizontal, and working about a vertical axis.

The squid bites through the fish from head to tail by a series of transverse bites. The alimentary canal of the fish is not eaten, but is left hanging down until the anus is reached, when the rectum is bitten through and the whole digestive tract of the fish violently rejected. After this, the meal proceeds steadily until the tail is almost reached. The morsel is then turned obliquely and the flesh bitten off the bones, first on one side and then on the other. (It is then that the twisting movements of the jaws are often seen.) The terminal fused vertebrae and the tail fin are finally rejected.

The duration of the meal is usually 15–20 minutes, but it may take longer if difficulties are encountered in getting rid of the head, or if the prey be unusually large.

Even while the head is being bitten off pieces of flesh can be seen passing down the oesophagus, in rapid procession, to the stomach. The latter becomes visible as soon as the first greyish food particles reach it, and immediately on their entry begins to contract violently, changing its shape rapidly and continually, becoming now oval, now spherical, or ballooning out, now on one side, now on another (Text-fig. 1B). As the meal proceeds and the stomach grows larger, the changes in shape become less marked, but the contractions continue to be plainly visible. By the end of the meal, when the tail vertebrae are rejected, the stomach is about a third of the length of the mantle and half its breadth; contractions cannot then be detected. Almost as soon as the meal is ended the stomach begins to diminish in size, and may become almost invisible (though not necessarily empty) after 2 hours.

At about the same time that the stomach becomes invisible the caecum, usually invisible, becomes a bright tan-brown; this colour remains in the caecum for about 2 hours. During that time the caecal sac appears to be fully

distended, and no contractions of its walls can be observed, though it is possible that slight contractions might be masked by the larger movements of the mantle. About 4 hours after the beginning of the meal the animal becomes, in general, indistinguishable from one which has not been fed. Shortly before the caecum becomes visible the liver often, though not invariably, appears darker and more swollen than usual; and when the caecum is coloured the liver matches it exactly, so that the two organs appear to be continuous. The liver returns to its normal appearance before the caecum; the changes in it are not so rigidly associated with the meal as those of the caecum, and may appear in a hungering animal.

The times given are only a rough indication of what appears to be the normal rate of digestion in captivity, a rate which can vary considerably. Of two animals fed at the same time with meals of the same size, the smaller animal digested its food more than half as fast again as the larger. In another brought into captivity with the stomach full of undigested food, digestion was suspended for at least 4 hours, and then proceeded at something less than the normal rate. This inhibition, which must have been the result of capture, illustrates the highly strung nature of these animals.

Investigations on newly decapitated animals showed that, in early stages of the meal, the stomach is a limp bag, filled with fluid containing lumps of food. Immediately after the lumps are swallowed these are squarish and clear-cut, measuring 2–3 mm. each way, and showing no sign of any rasping radular action or of previous digestion. These lumps are rapidly broken down and the liquid in the stomach becomes filled with flocculent grey masses of digested food and, if a fish has been taken, with a glittering suspension of silvery particles from its scales. As digestion proceeds this suspension disappears, the flocculent matter diminishes in amount, and the contents become more and more compact, until only a mass remains of perfectly clean and transparent skeletal parts of crustacea or fish. These are not necessarily ejected before the next meal, and may be found present with newly swallowed food.

In decapitated animals there may take place a sort of reversible peristalsis, up and down the sac, as well as the violent churnings observed during the meal.

The whole organ can also contract violently, emptying its contents; in hunger it goes into a state of tonic contraction. Death may overtake the stomach when in active contraction and, as a result, stomachs of varying shapes may be found in material which has been dead some time. There can be no doubt that these forms represent what are, in life, but transitory phases of activity. The shape of the stomach in freshly killed material is always a smooth oval.

The caecum of a freshly killed hungering squid is always partly or fully distended and full of colourless liquid, sometimes slightly milky, usually perfectly clear. Within 35 minutes of the beginning of the meal a yellowish or pinkish tinge appears in the liquid, which may also contain a few small, often glittering particles. As the meal proceeds the colour of the caecal liquid deepens in intensity, until it is visible in the living animal (see above); the suspension of

particles also thickens and then disappears; there is some evidence that at least one later influx of particles takes place and is again removed. During the latest stages of the meal, when absorption is proceeding, the caecal contents are milky and yellowish and free from particles. When the meal is ended the caecal contents return to the colourless, limpid condition associated with the hungering condition (Text-fig. 1). No large food masses have ever been observed in the caecum: on this all authors are agreed (Bourqelot, 1885; Williams, 1909; Sellier, 1907, 1910).

It may be assumed that the colour which appears in the caecum during the meal represents hepatic secretion, since the brown of the caecum matches that of the liver exactly (p. 10); a yellowish or brownish colour has never been seen in the stomach.

The suspension of particles comes, as its appearance strongly suggests, from the stomach, and its passage has been observed in decapitated specimens; this was confirmed by a specimen which was killed 35 minutes after feeding, and showed, when sectioned, particles of carmine from the food free within the caecum. (Ample evidence for the penetration of food into the caecum was also obtained from the morphology, the injection experiments (pp. 18 ff.), and histology (pp. 25, 26), which revealed cells of the caecum crammed with absorbed fat.)

The intestine is sometimes found to contain half-digested food, which was certainly prematurely ejected from the stomach at capture or death; it is also often found distended with liquid resembling the caecal contents.

These observations on the living and newly killed animal confirmed those of Williams on *L. pealeii* (1909, p. 44) in so far as the action of the jaws is concerned, and showed that breakdown of the food, to particulate form at least, takes place in the stomach and that liquid and particulate food is then passed into the caecum. It remained to determine where digestion is completed and where the digested food is absorbed: whether in the intestine as maintained by Bourquelot (1885, p. 69), in the caecum, as supposed by Williams (1909, p. 39), or in the liver and caecum, as claimed by Cuénot (1907, p. 237) and Sellier (1910), and where and in what manner the digestive enzymes are produced. Evidence on these questions and details of the mode of action of the various organs were obtained by examining the gross structure and histology of the digestive system, both during the meal and during hunger and starvation.

The Structure and Action of the Digestive System

The Buccal Mass

The general arrangement of the digestive organs is shown in Text-figs. 1 and 2. The buccal mass with its inner and outer circular lips is freely movable and even protrusible, and is surrounded by a well-developed, sucker-bearing buccal membrane (cf. Naef, 1923, p. 179, Text-figs. 77, 78). The jaws are well developed and powerful. The radula is present, and it and the jaws are

figured by Naef (loc. cit., Text-fig. 14, Pls. 14, 17). It will be seen from the description of the meal (p. 9) that the food is swallowed too quickly for any rasping action of the radula to take place: this agrees with the texture of the radular teeth, which are delicate, flexible structures, pale gold in colour, and presenting the strongest possible contrast to the hard, dark jaws. The radula of *Loligo* is a tongue, used in swallowing like the rough tongue of the cat; the rough surface may in some cases be used, as Williams said (1909, p. 44), 'upon resistant objects'. Such an interpretation was already put forward by Owen (1836, p. 532) and by Cuvier (1805, p. 345), who described the movements of the radula, which raises and lowers the teeth 'et passe insensiblement les aliments dans l'oesophage'.

The Glands of the Fore-gut

No detailed study of these organs has been made by the present writer.

The 'anterior salivary', 'buccal', or 'mandibular' gland is small, and lies partly applied to and partly within the buccal mass (Wülker, 1910); the 'posterior salivary' or 'poison' gland, which arises as a paired structure but, in *Loligo*, is single in the adult, lies partly embedded in the anterior end of the liver (Text-fig. 1c) and is connected with the buccal mass by a long duct, running parallel with the oesophagus. The function of the small buccal gland is unknown. Williams (1909, p. 34) found no lipase nor amylase and suggested a proteolytic action. It seems probable that the chief function of this organ is to provide the lubricant necessary for swallowing.

The poison gland has been shown by Bottazzi (1916) and by Bottazzi and Valentini (1924) to secrete tyramine, which poisons the prey, and by Sereni (1928) to have an endocrine effect on the tone of the squid's own muscle. Although most of the results were obtained on *Octopus* and *Eledone*, sufficient comparative data exist to justify extending these results, in this case, to *Loligo*.

Accounts of structure of the buccal gland have been given by Wülker (1910, p. 44, Figs. 43, 44, 45) and of both the buccal and the poison gland by Joubin (1887).

The Musculature of the Digestive Tract

The whole of the alimentary canal is lined with a continuous epithelium, whose cells and their nuclei (generally in the middle of the cell) vary greatly in shape with the degree of contraction of the organs. The lining epithelium of the oesophagus and stomach is cuticle-secreting, that of the caecum and intestine ciliated. The basement membrane is always in intimate connexion with a surrounding network of connective tissue, by which it is separated from the muscular coats with which the alimentary canal is everywhere supplied. These muscle layers are in turn surrounded by a second layer of connective tissue covered by a pavement epithelium which, in the stomach and caecum, is part of the coelomic lining. In the stomach and caecum, layers of connective tissue are interspersed with the layers of muscle-fibres. The connective tissue forms a delicate mesh-work with big, oval nuclei; nerves and

blood-vessels run through it, and strongly developed connective-tissue fibres with long, very slender nuclei; these fibres are seen especially well developed immediately under the lining epithelium. (Gariaeff (1915) states that these are too fine to be easily seen, but after staining with Prenant-Hollande their brilliant green colour makes them easily visible.) The muscle-fibres show the characteristic structure described by Marceau (1904, 1906), i.e. a central core of lightly staining cytoplasm, surrounded by a more darkly staining fibrillar sheath. This sheath consists of very fine longitudinal fibres, arranged parallel to the long axis of the cell or slightly twisted round it and thus showing none of the transverse striation due to close twisting described by Marceau (1905) for the mantle cells of *Loligo vulgaris* and *Sepia officinalis*, and for the mantle and heart cells of *Octopus vulgaris* (but not found by him in the arm muscle-fibres), and plainly visible in the heart cells of *Loligo*. Only in one region of the muscular part of the hepatic duct (pp. 32, 36) some fibres are to be found, lying between the lining epithelium and non-striated fibres, which have the striated appearance. All the muscle-cells are very long, so that nuclei but rarely appear in the sections. Gariaeff describes spiral striation in the muscles of the alimentary canal, but the present writer has not been able to confirm this.

The Oesophagus and Stomach

The *oesophagus* (Text-fig. 1) lies in a groove between the liver and pen, and passes through the liver in an obliquely ventral direction to open into the stomach. It is a muscular organ by whose active peristalsis the fragments of food are passed rapidly from mouth to stomach. There is no crop.

The musculature consists chiefly of a well-developed layer of circular muscles; the inner longitudinal muscle-fibres are for the most part confined to a series of longitudinal strands, which are reinforced by connective-tissue fibres, show as faint ridges in the dilated organ, and, in the contracted state, form deep folds obliterating the lumen of the oesophagus. Nerves and blood-vessels run in both of the connective-tissue layers. The great depth of connective tissue figured by Gariaeff (1915, Pl. IV, fig. 48) between the lining epithelium and muscle layers has not been found by the present writer.

The *stomach* (Text-fig. 2) is a very muscular sac, lying on the animal's right side, with the oesophagus and intestine opening side by side into the anterior end. The muscles (whose action is described on pp. 9, 10) are arranged in wide, interwoven bundles, many of which run obliquely; the mesh-work of connective tissue in which the blood-vessels run penetrates the muscle layers and surrounds and separates the bundle of muscle-fibres.

Both oesophagus and stomach are lined with a soft, smooth, colourless, distendable cuticle. Miss C. H. Brown kindly examined the fresh cuticle of *Loligo* and reports as follows:

'Millon's, the xanthoproteic and ninhydrin tests for proteins all gave positive results, indicating the presence of proteins in the membrane. The lead acetate test for sulphur was also very slightly positive.

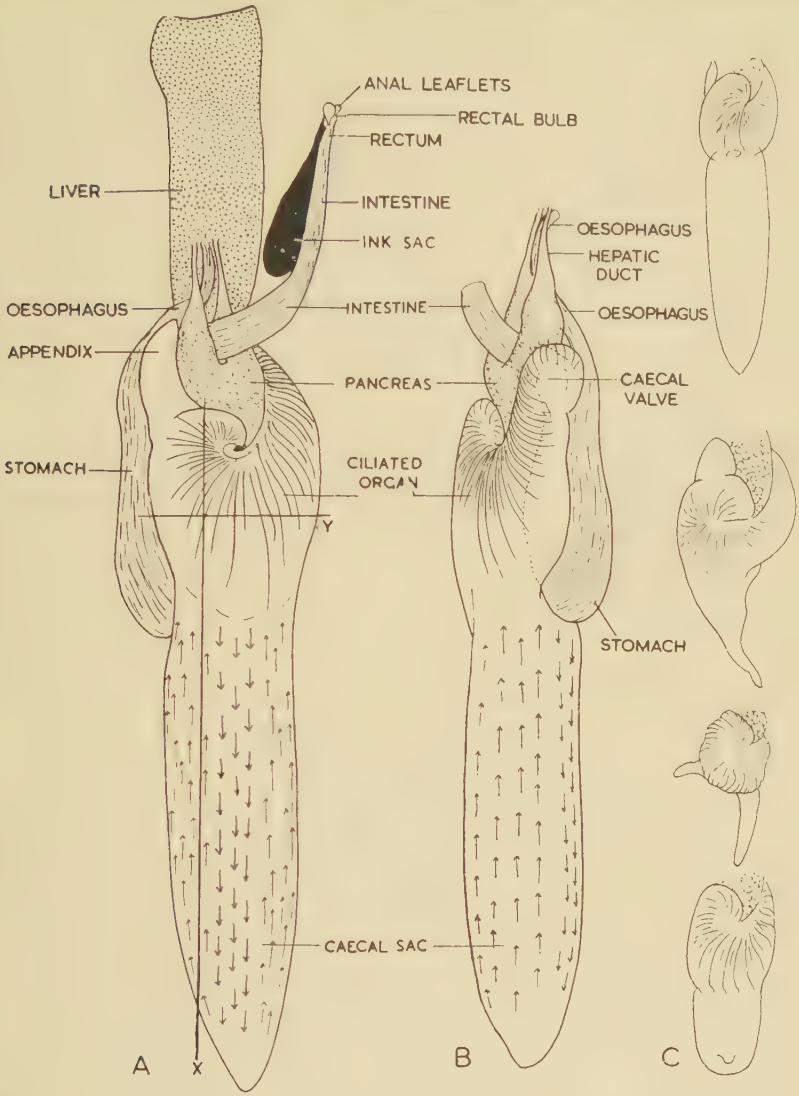
'The material would not dissolve in boiling water but thickened and contracted. Dilute acid had no apparent effect, but concentrated hydrochloric acid dissolved the membrane completely. On boiling in dilute or concentrated sodium hydroxide solution there was always an insoluble residue and this residue gave a positive chitin test with iodine followed by dilute sulphuric acid. Swelling agents such as lithium iodide and urea caused the membrane to swell slightly but not dissolve. The argentaffin and diazo tests for polyphenols in the membrane were negative.

'It is concluded, therefore, that the stomach lining of *Loligo* is composed of a chitin protein complex.'

The cuticle varies greatly in thickness with the state of contraction of the organ; this variation is much greater in the stomach than in the oesophagus. In the distended stomach the cuticle is about 10μ thick; in the contracted stomach the cuticle is both greatly thickened (reaching, in the adult organ, a thickness of 350–400μ) and thrown into rounded folds, which become sharp and hard after most fixatives. Similar ridges in other cephalopods have been described as 'grinding ridges': in *Loligo* the observations on the living and freshly dead animal show that the action of the stomach is here a churning, not a grinding action, and, indeed, the ridges are only fully developed when the stomach is empty, or contains only indigestible residue. This residue (p. 10) consists of sharp, hard fragments of bone and chitin, and the thickened, contracted cuticle will afford the gastric epithelium the same protection as can be given to the intestine during defaecation by copious mucus-secretion (p. 27).

The lining epithelium of both the oesophagus and stomach shows no sign of activity other than that of cuticle-secretion. The histology of the two epithelia is closely similar. The cells are cubical to columnar, according to the state of contraction, and the cytoplasm finely fibrillar. The fibrillae are continuous with the basement membrane, and through it are in such intimate contact with the underlying connective tissue that sections have been found in which the cells, torn away by the knife, left their fibrillae adhering, brush-like, to the underlying layers. In the stomach these fibrillae extend through the whole depth of the cell and appear to be continuous with a fibrillar formative layer which is present everywhere between the epithelium and the finished cuticle. At times, indeed, the cell-border disappears altogether, and the intra- and extracellular fibrillae can only be distinguished by the acidophil staining of the formative layer (Pl. I, fig. 5). The finished cuticle appears to be formed of two substances: one, the acidophil fibrillar sheet, the other represented by irregular, basiphil masses (staining red with Mallory), sometimes visible within the cell, sometimes just outside the cell-border, sometimes amongst the strands of the fibrillar layer (Pl. I, figs. 3, 4). This double method of secretion may be compared with the mixed nature of the cuticle found by Miss C. H. Brown and indicated by a rather muddy acidophil staining often shown by the cuticle. (The formative layer is described and figured by Gariaeff for other Cephalopod genera (1915, p. 73, Pl. VIII).)

In the oesophagus the formative layer is not always present, and, when present, is very narrow. The double staining found in the gastric formative



TEXT-FIG. 2. A, B. Mid-gut and mid-gut glands of *Loligo*; A, ventral view, B, seen from the animal's left side. The arrows mark alternative systems of ciliary currents in the caecal sac (see p. 22). (Lines X and Y mark planes of section of Text-figs. 3A and 4A respectively.)

C. Four sketches of the caecum showing post-mortem variations in size and shape—all drawn to the same scale.

layer has occasionally been seen in that of the oesophagus, and basiphil granular fragments occasionally detected within the cells. When the formative layer is absent, the cells have a very narrow but brightly acidophil cell-border. The fibrillar layer cannot be traced to the cell-border (this is confirmed by Gariaeff, 1915, p. 49) and there is no visible connexion between it and the cuticle. This may be a qualitative difference; the other differences are probably purely

quantitative, representing a lower rate of secretion. This difference may b related to a very much lower rate of wear and tear in the oesophagus than i the stomach: food passes rapidly along the oesophagus, and at a stage at whic the sharp parts are still largely protected by flesh. Since, however, the oeso phagus is ectodermal, the stomach mesendodermal in origin (Ranzi, 192 p. 93), some qualitative difference between the two epithelia is not wholl improbable.

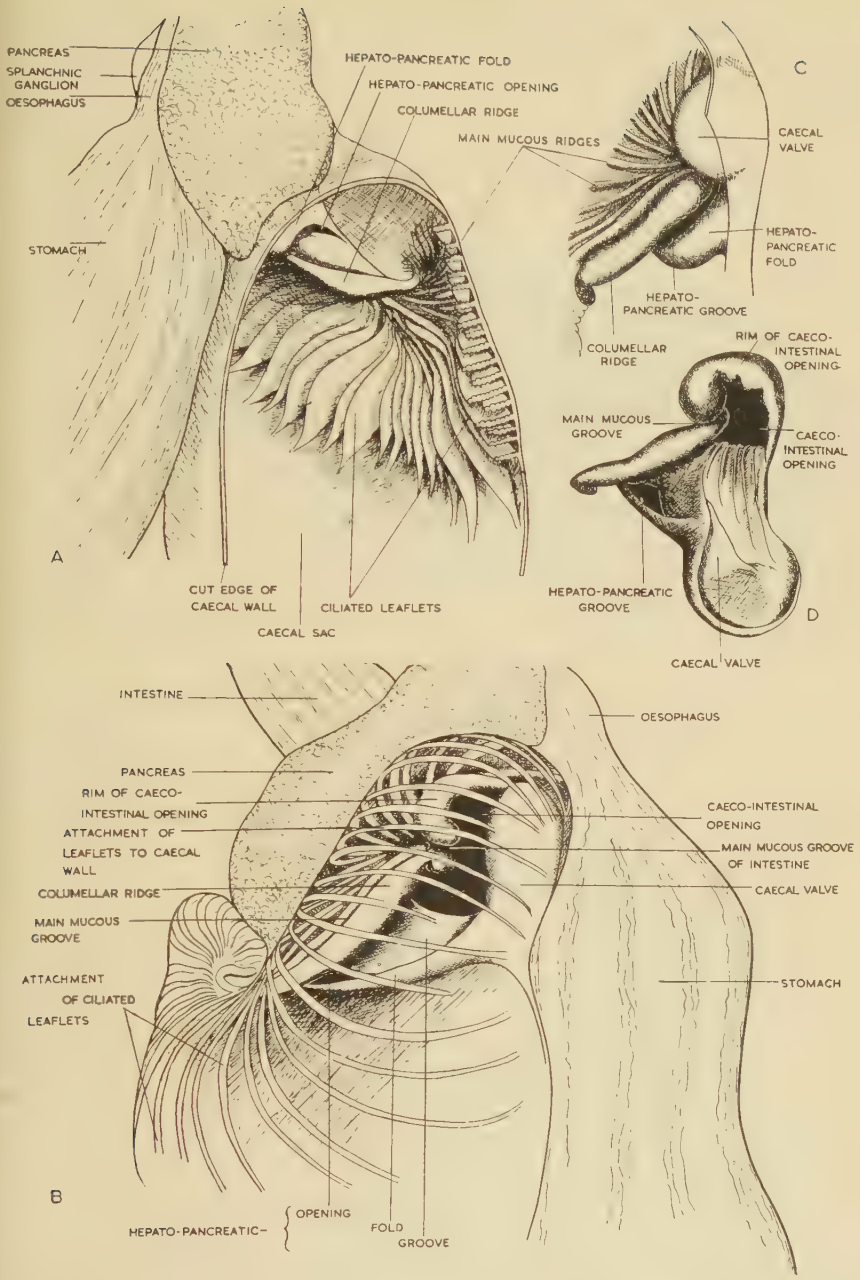
The oesophagus is thus purely a passage: the stomach is a place of pre liminary digestion where, under the action of enzymes formed elsewher (p. 36), the food is broken down to particulate form and separated very com pletely from larger indigestible residue. The only gastric secretion is that o the protective lining cuticle.

The Caecum

The caecum has three activities: first, as has long been recognized, it is th ante-chamber between the mid-gut gland, which is the source of digestiv enzymes (p. 31), and the stomach, where the preliminary digestive breakdow takes place (p. 10); secondly, it is, as will be shown, the place of final digestio and of absorption; thirdly, associated with its absorptive function, it contain an elaborate ciliate and mucous collecting mechanism whose function would seem to be to clear the nutrient fluid contents of all solid particles. These three independent functions modify and complicate the internal anatomy o the organ, which is illustrated in Text-figs. 3 and 4.

The caecum of *Loligo* consists of two parts: an anterior, spiral portion, o about one and a half turns, and a long sac, blown out, as it were, from the posterior side of the spiral (Text-fig. 2). The spiral portion has its axis obliquely dorso-ventral, and the last half-turn is separated from the rest by the wedge-shaped pancreas (p. 32) whose thin end runs in the columella o the spiral and opens at its apex; the caeco-intestinal opening is at the othe end of the spiral, at the mouth, as it were, of the snail-shell. These two openings are connected by a groove, the 'hepato-pancreatic groove', which runs round the columella of the spiral, and is bounded on one side by a well-marked ridge, the 'columellar ridge', and on the other by a fold, the 'hepato-pancreatic fold'. This fold can shut down on the columellar ridge, and convert the hepato-pancreatic groove into a tube, leading direct from the hepato-pancreatic duct into the intestine.

The ventral surface of this part of the caecum is set with a number of leaf-like ciliated folds, projecting across the spiral, and alternating in length, so as to define a system of converging 'interleaflet grooves', all ultimately leading into a 'main mucous collecting groove' (Text-figs. 3, 4), which runs round the columella, parallel to the hepato-pancreatic groove, and separated from it by the columellar ridge. The leaflets are themselves ridged and grooved with 'primary' and 'secondary' leaflet grooves (Text-fig. 5; Pl. II), so that each inter-leaflet groove is itself a collecting groove for the two leaflet faces which bound it, and the whole forms a ciliate and mucous collecting mechanism, whose



TEXT-FIG. 3. A. Anterior end of caecum (plane of section shown by Text-fig. 2A, *X*) showing hepato-pancreatic opening.

B. The same, showing the caecal valve; the ciliated leaflets indicated by their bases only.

C. The caecal valve shut. D. Open.

A, B, drawn from living post-larvae.

action is described on pp. 22 ff. Williams (1909, p. 36) described the arrange ment of the leaflets in *L. pealeii*, but regarded them as radiating from, nc converging towards, the columellar ridge and caeco-intestinal opening Gariaeff (1915, p. 51) regarded them as glandular. Where the form of th spiral is unaffected by the sac, the leaflets run across the spiral, so that thei lines of attachment, seen from the outside, resemble the lines of growth o a snail-shell.

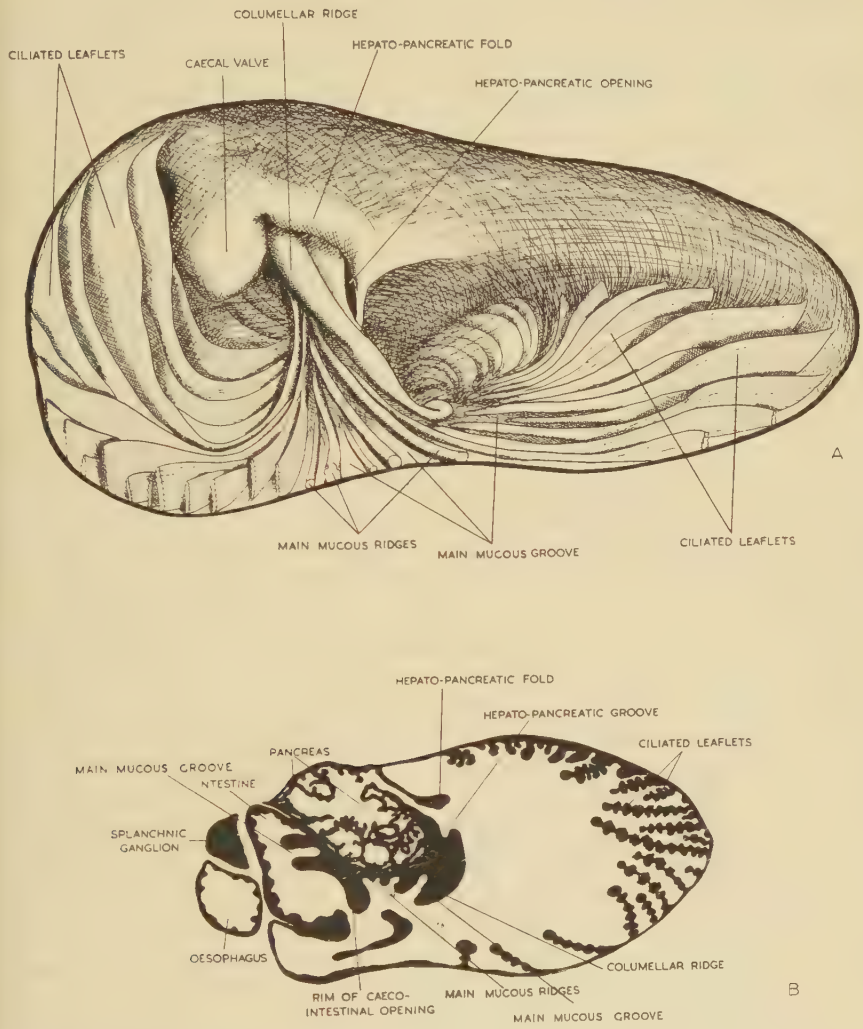
The caeco-intestinal opening is protected by an elaborate closing mecha nism: the hepato-pancreatic fold is expanded at the opening into a saucer shaped lid, and the opening has a thick rim or lip on to which the lid can clos (Text-fig. 3). This rim curls round where the columellar ridge passes out o the caecum, and forms with it the main mucous groove in the intestine (p. 27 Text-fig. 4). As a result, the lid shuts down across the columellar ridge anc groove, without blocking the latter, which opens freely into the intestine, eve when the lid is closed (Text-fig. 3). Moreover, since the lid is continuous wit the hepato-pancreatic fold, then when the lid is closed on to the rim and th hepato-pancreatic fold closed down on to the columellar ridge, the hepato pancreatic groove is shut off from the caecum but communicates freely wit the intestine and stomach. It seems probable that the fold and lid act together so that the hepato-pancreatic duct is only open to the caecum when the caeco intestinal aperture is also open (cf. p. 39).

The action of the valve and fold thus controls the movements of the caeca contents and the passage of enzymes into the caecum, but not the action of th ciliated organ, nor the passage of the enzymes from the mid-gut gland to th stomach. Its function also appears to be to exclude large food particles fro the caecum: masses of debris are often to be found accumulated against th intestinal face of the valve although never found in the caecum (p. 11). It i not easy to envisage how this exclusion takes place, unless contact betwee solid food and the valve or rim stimulates the valve to close.

Both valve and rim are solid structures, reinforced by cartilage, and th valve is muscular and under nervous control and richly supplied with nerve from the splanchnic ganglion (Alexandrowicz (1928) and personal observation with methylene blue). A sphincter-like muscle runs in the edge of the valve and can tie it down over the rim, as the cover is tied down over the rim o a jam jar. A valve which had been cut off from the caecum showed slow contractions across the valve and round the rim, showing that the closing action of the valve is due to intrinsic musculature. The valve is attached to th wall of the caecum (Text-fig. 3), so that, when valve and caecal muscles are both relaxed, and the anterior part of the caecum distended, the valve is held widely open, and fluid can pass freely in or out of the caecum. Alternatively it can act as a simple valve, allowing ingress to but not egress from the caecum or, fastened down over the rim, it can isolate the caecal contents completely Observations suggest that all three alternatives are employed during the digestive cycle.

Injections of carmine in sea-water into the gut, through the mouth, demon-

strate this complex action. Out of thirty-seven injections, thirty-three entered the caecum readily, sometimes more readily than the stomach. In four animals carmine failed at first to enter the caecum, but entered it



TEXT-FIG. 4. A. Anterior end of caecum of adult *Loligo* viewed from the sac-plane of the section shown in Text-fig. 2A.

B. T.S. of the same region of a young *Loligo*, passing through the caecal valve.

later; this, in view of the successful injections, could not be due to a simple valve-action, but must rather be due to muscular action of the caecal valve. Finally, in a small specimen whose stomach was still actively contracting, the contractions were seen alternately to pump food into the caecum and to suck it back again, which could only take place if the valve were held widely open.

The presence of the valve has been described by Bourquelot (1885, p. 67). Williams (1909, pp. 36–8, Pl. I, fig. 2, Pl. III, figs. 18 and 19) gives a detailed account of it in *L. pealeii*, where the valve is present, but with an extra protection to the hepato-pancreatic opening which it is hoped to discuss in a later paper. According to Bourquelot it is mentioned by Cuvier (1838), but the passage, which was posthumously inserted by Dunervois, appears to refer to the main mucous ridges. Bourquelot's interpretation of the valve in *L. vulgaris* is in direct disagreement with mine. For him the valve opened into the intestine, acted as a simple flap-valve, and could be applied either to the caecal opening to prevent the entry of food passing from the stomach to the intestine, or to the intestinal opening to prevent the entry of digestive juices, passing from the caecum to the stomach. Williams appears to have misinterpreted Bourquelot's account, for he says: 'All these facts lead us to the following conclusion *which agrees with that of Bourquelot* . . . that the food is comminuted and partly digested in the stomach . . . [and] that the partially digested food then passes into the caecum for complete digestion and absorption' (Williams, 1909, p. 38; the italics are mine). Bourquelot's own words are: 'Ainsi donc la digestion se fait tout entière dans l'estomac. . . . Les aliments ne passent pas dans le caecum intestinal: une disposition anatomique spéciale s'y oppose' (Bourquelot, 1885, pp. 68, 71). For Bourquelot the caecum was thus a sort of enormous gall-bladder. In a large number of animals examined by the present writer no trace of the caecal valve has been seen in the intestine from which the whole apparatus is completely invisible.

The caecum further possesses, at the level of the ciliated organ, a triangular pocket, the 'appendix' (Text-fig. 2), which, like the sac, shows great variations in size and shape.

Thus, as already stated, the caecum of *Loligo* consists of two parts: the anterior part, containing the appendix, the ciliated organ, the openings from the mid-gut gland and intestine, and all the complicated structures associated therewith, and the posterior, simple sac. The whole organ is muscular and lined with a ciliated epithelium which, in the ciliated organ, is interspersed with mucous cells.

The caecum of a freshly killed squid is always relaxed and inert. The irritation of dissection and handling the animal, however carefully, often starts contractions of the caecal sac, either gentle waves of contraction, up and down the sac, stirring and mixing the contents, or a more violent pumping driving out the contents, or slow tonic contractions which may ultimately reduce the sac to a tiny, thick-walled, finger-like process (Text-fig. 2c). In trawl-material such a minute sac is not infrequently found, but, as it has never been found in freshly killed material, it should probably be regarded as a purely post-mortem effect, similar to the distorted conditions found in the stomach in the same material (p. 10). In some animals the volume of the sac is somewhat restricted by ripe gonads or by an unusually full stomach, and the appendix may then be greatly enlarged, suggesting that its function is to compensate at times for a constricted sac. Sometimes

the appendix shows active pumping movements, which violently stir its contents.

The sac can be partly cut off from the ciliated organ by a diaphragm-like sphincter (Text-fig. 2c), or by local contractions of the whole anterior region of the sac-wall.

The ciliated organ can contract as a whole, and rhythmic contractions have been seen passing round the spiral. Individual leaflets show slow transverse twitches, which open and close the leaflet grooves and can go on for some hours after the caecum is dissected out. Longitudinal tonic contraction of the leaflets may be due to intrinsic leaflet musculature, or to the general musculature of that part of the caecal wall.

Ciliary Currents of the Caecum

Most of the animals used for observing the ciliary currents had died in the trawl; observations were confirmed wherever possible on decapitated specimens. In general a suspension of finely ground carmine in sea-water was used to determine the currents. The mantle-cavity was opened from the ventral side and the caecum exposed. Carmine was injected through the mouth, until it just appeared in the caecum; a hypodermic syringe was used, with a large needle whose point had been ground away so as to minimize the risk of piercing the thin wall of the oesophagus. The currents were observed through the wall of the caecum, without further dissection. If it was desired to re-examine the animal, after an interval, the whole animal was left under sea-water circulation until required again.

The ciliated organ and caecal sac were also opened and examined independently under the microscope and binocular dissecting microscope, with and without the use of carmine. A dissecting dish was made by lining a flat-bottomed glass dish with paraffin wax, so as to leave a clear space in the middle; and the sac was thus examined by transmitted light.

The currents in material which had died of shock in the trawl (p. 4) were enormously slower than in decapitated material. This had its advantage in the observation of detail, but a disadvantage in the resulting accumulation of mucus, which tended to block the action of the ciliated organ altogether. The cilia of the ciliated organ in 'shock material' were almost invariably inactive when the injection was made, and only began to take effect after 1–3 minutes. This inactivity was never found in decapitated material: it may be related to the fact that trawl-material was inevitably only examined several hours after capture, during which time the cells of the close-set leaflets would become increasingly short of oxygen and under the influence of their own metabolites.

The cilia of the sac act without mucus; particles carried in the currents move freely with respect to each other. Save for a narrow transverse tract, immediately posterior to the ciliated organ, the beat of all the cilia is parallel to the long axis of the sac. In the most usual condition observed, the transverse current gathered particles into a narrow ventral tract which beat into the sac, sweeping particles away from the ciliated organ. The currents of the rest of

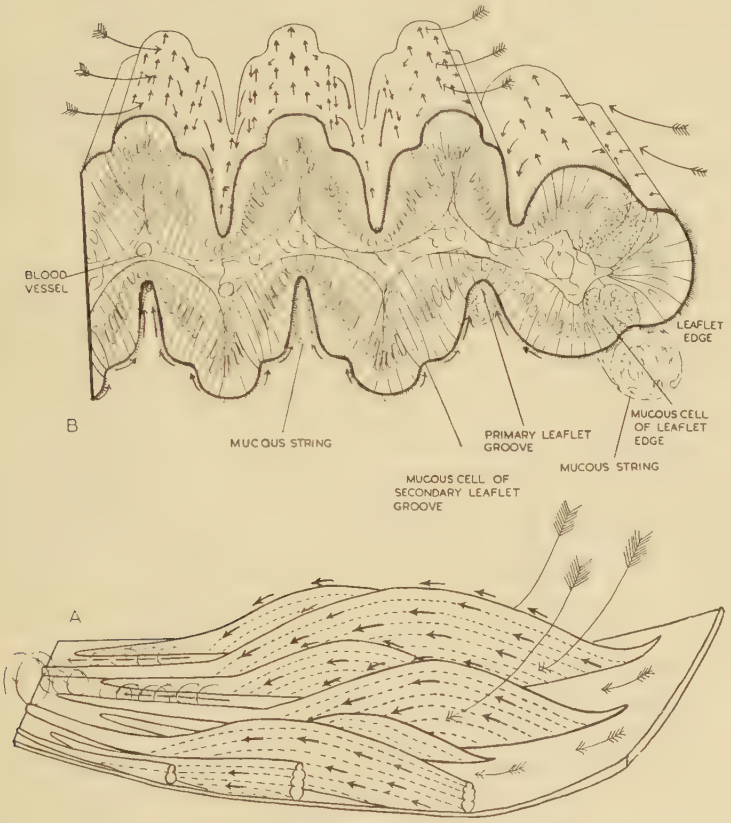
the sac lining were usually directed out of the sac (Text-fig. 2A). In one or two specimens, however, the ventral 'in' current was absent, and in some a corresponding dorsal current was observed (Text-fig. 2B). In one specimen at least, the 'in' and 'out' tracts were of equal width.

These observations must be interpreted as reversals of ciliary current within the sac. Normal individual variations of this magnitude are foreign to all our experience of ciliary tracts; nor can action at a distance, by opposing tracts, on a sheet of mucus, be here in question, since the sac is free of mucus. Again, we cannot postulate here a change of direction effected, as in other molluscan ciliary systems such as the labial palps of lamellibranchs (Kellog, 1915; Yonge, 1926), by opposing tracts on ridges and grooves, whose effect may be controlled by opening or closing the grooves through muscular action. The currents in the sac are all parallel to its long axis, but the sac-wall contracts, not into parallel folds, but into a network of fine wrinkles. The significance of this reversal is hard to find, for in view of the sac's muscular activity (p. 20) the cilia of the sac would seem to function simply as stirrers of the whole contents when the muscles of the sac are completely relaxed; of the layer immediately next the lining epithelium when the muscles are active (a layer which, owing to the friction between it and the epithelium, is but little affected by mass movements of the caecal contents). In either case, any of the systems of tracts described above would appear to be equally effective.

The *ciliated organ* has a mode of action and effect entirely different from that of the sac; its cilia act with mucus, and gather up all particles within range (nutritious and otherwise) into a number of mucus-strings, which are gradually twisted together and passed into the main mucous collecting groove (Text-figs. 3, 4) and so out of the caecum, along the intestine, to the anus. The details of the tracts are shown in Text-fig. 5. The apical cilia of the leaflet ridges combine with cilia of the caecal wall between the tips of the longest leaflets to set up a general drift towards the caecal opening. With the exception of two narrow opposing tracts on the shoulders of each leaflet ridge, the rest of the cilia on the ridges beat down into the 'primary' leaflet grooves, carrying particles from the food and balls of mucus secreted by the cells of the 'secondary' leaflet grooves (p. 16, Pl. II). In the depths of the groove, particles and mucus combine into a mucus-string, which is swiftly carried towards the junction of each leaflet groove with the inter-leaflet groove, i.e. towards the columella of the spiral. The two opposing tracts on the shoulder of the ridges are so narrow that particles often miss them altogether. Sometimes, however, these tracts set up eddies and delay the entry of particles into the grooves. An aggregate of particles, caught in this eddy, will be spread out into a thin line before being swept down into the groove, thus reducing the risk of the groove becoming choked with large masses of particles. The whole action is normally very swift, and carmine appears in the inter-leaflet grooves an astonishingly short time after its injection into the caecum; but, once the leaflet grooves have become choked by a large mucus-string, the cilia of the grooves become powerless.

Especially thick mucus-strings are found in the grooves on each side of the leaflet edges (Text-fig. 5B). The size of these strings is probably due to the specially viscous nature of the mucus which forms them (p. 26).

The collecting-ridge currents are similar to those of the leaflet edges, with which they are continuous. The cilia of the collecting grooves must have a rotating action, as the strings in the larger grooves are found to be complex



TEXT-FIG. 5. Ciliary currents of the caecal leaflets. A. Portion of caecal wall, and attached leaflets. B. Solid section of the edge of a single main leaflet. Water-currents shown by feathered, mucus-currents by plain, arrows.

ropes of many strands, twisted together. The final result is passed out by the main mucous groove of the caecum into that of the intestine (p. 27) and so to the anus.

The caecal valve is covered with a ciliary-mucous-epithelium and particles impinging against its caecal surface are swept in a sheet of mucus round the edge to the intestinal surface.

Those particles which escape the cilia of the leaflet edges are caught into the leaflet grooves, and those which escape the mucus of the leaflets are caught by the collecting ridges and sent down into the collecting grooves, or even into

the main groove itself; and, should any still escape, they are swept against the caecal valve, and become entangled there in a mucus-sheet, in which they are swept round the edge of the valve into the intestine.

There is no sign, in all this elaboration, of any sorting mechanism. There is no evidence that, even in the thicker strings formed at the free edges of the leaflets, larger particles are entangled than those in the leaflet grooves. Further were it so, there would still be no real sorting, since all strings are ultimately twisted together into the same rope. A sorting mechanism is scarcely to be expected in a mechanism purely active in removing waste and dealing with particles of small average size: only particles 10μ and less in diameter are normally to be found in the mucus-strings: the ciliated organ is purely a collecting mechanism, which removes all solid particles within its range out of the caecum into the intestine. Some food particles are caught into the strings before they can be digested in the sac: this is the only wastage of assimilable food in the whole digestive mechanism (cf. p. 10).

The significance of the leaflet currents would seem to be to clean the apical and latero-frontal regions as quickly as possible, sweeping all particles down into the leaflet grooves where, unless the mucus-strings are abnormally thick, they are deeply buried, so that the upper parts of the ridges are left free for absorption (p. 26, Text-figs. 5B, 6E).

The caecum thus may be regarded as two separate organs which can, in fact, be partially isolated from each other (p. 21). The sac is an organ of final digestion and absorption, its muscles and cilia combine to change and stir its contents; the ciliated organ, hepato-pancreatic groove and fold, and the caecal valve give the anterior part a complexity of structure corresponding to the multiplicity of its function.

The Histology of the Caecum

The arrangement of the muscle layers in the caecal sac is the reverse of that in the oesophagus and intestine: the inner layer is of circular, the outer of longitudinal muscle-fibres. The two muscle layers are separated from each other, and from the lining epithelium and covering peritoneum of the caecum, by layers of delicate connective tissue, set through with connective-tissue fibres. These fibres are particularly plentiful between the circular muscle layer and the lining epithelium of the sac. The middle layer, which lies between the two sets of muscle-fibres, and which has no equivalent in the oesophagus or intestine, contains a rich blood-supply, corresponding to the organ's absorptive activity. The outermost connective-tissue layer contains scattered muscle-fibres running in all directions, as well as connective-tissue fibres, some of which, running radially, penetrate the layer of longitudinal muscle-fibres.

In the ciliated organ the orientation of the muscle-fibres is difficult to trace: there is some evidence that the inner layer runs across, the outer layer round, the spiral. Connective-tissue fibres are particularly well developed immediately below the ciliated epithelium and reinforce the ciliated leaflets, in which Gariaeff (1915, p. 52) has described isolated muscle-fibres. The leaflets

are well supplied with blood-vessels, lying, as in the sac, in a layer of connective tissue in between the two muscle layers.

The caecal valve, columellar ridge, and main mucous ridges are all reinforced with cartilage, apparently indistinguishable from vertebrate cartilage.

The sac of the caecum is lined with a ciliated epithelium, whose cells show, according to the degree of contraction of the surrounding muscular coat, every form from pavement to deep columnar epithelium. The cilia have basal granules, and parallel rootlets penetrating a short distance into the cytoplasm; they are not always present in sections, but their action is always readily demonstrable, even in animals which have been dead for some hours, and at all stages of digestion. The nucleus is oval, with one or more nucleoli, and varies in proportions with the changing proportions of the cell. The cytoplasm appears homogeneous or finely granular. A small acidophil, or faintly basiphil granule occasionally appears between the nucleus and the cell-border, which is well stained with eosin in Prenant-Hollande preparations.

The cells show typical fat-absorption pictures after treatment with osmium tetroxide (Text-fig. 6F), pictures which must represent absorption after complete extracellular digestion. There is no histological evidence of secretion by the lining epithelium of the sac.

The ciliated cells of the ciliated organ are columnar cells, whose individual variations in height assist in the definition of the leaflet ridges and grooves. Alone in all the lining cells of the alimentary canal, these cells do not fluctuate in shape. The cilia are strongly developed, and show basal granules and parallel rootlets, which have been traced half-way between the free edge of the cell and the nucleus (Pl. II). The nucleus, in the middle of the cell, is large and oval, with one or more small nucleoli. The cytoplasm is diffusely granular after all fixatives used, and shows great variation in depth of staining. This is partly, at least, related to the age of the cell: the extrusion of worn-out cells, narrow and very darkly staining, appears not infrequently in sections, but it seems possible that these variations may also represent a rhythm of secretion; the palest cells have a slightly distended appearance, and the cytoplasm distal to the nucleus one consistent with accumulations of diffuse secretion, possibly of mucus-secretion, possibly connected with the enzyme system. The actual secreting surface is much less than that provided by the mid-gut gland, but an activator might be produced by the ciliated leaflets, and this was in fact claimed by Henri (1903) for the leaflets of *Sepia officinalis*, and has been confirmed by Romijn (1935, p. 413). Replacement of the worn-out cells is supplied by cell-divisions, whose mitotic figures have been observed situated close under the cell-border (Text-fig. 6C) as described by Gutheil (1911, Figs. 5–16) and Saguchi (1917, p. 257, Pl. I, fig. 19). A small body appears in the distal half of many cells, chiefly those of the lateral ridges (Text-fig. 6D, Pl. 2). It appears, after fixation with either Helly or Flemming-without-acetic, to consist of an imperfect, basiphil, spherical shell, resembling a unit of the Golgi apparatus, and arising near the nucleus. It apparently moves up the cell, changing as it nears the cell-border into an irregular basiphil mass,

or breaking up into a number of basiphil granules, and is finally extruded. The cells of the ciliated organ show a marked rhythm in regard to this structure: cells of one ridge showing very closely the same phase, and of a whole leaflet, less exactly so; this same rhythm of individual leaflet ridges is also traceable in the occurrence of the mitotic figures. The cilia of the cells of the apical ridges appear to have shorter rootlets than those of the lateral ridges; among the apical rootlets, clusters of basiphil granules may be seen (Pl. II).

The ciliated cells of the ciliated organ do not show the same active absorption shown by those of the sac or of the intestine. The ciliated organ of an animal whose sac and intestine were in active absorption showed fat-droplets chiefly in the cells of a few leaflet ridges near the caeco-intestinal opening; these fat-droplets were very fine and scattered evenly through the upper part of the cell (Text-fig. 6E).

The mucous cells of the ciliated organ are of two types. One, forming the secondary leaflet groove, is a slender cell, staining darkly with haematoxylin in preserved material, readily with methylene blue in life, and extruding a basiphil ball of mucus. The second does not take up methylene blue in life, gives the typical blue stain with Mallory, and green with Prenant-Hollande; the unripe cell is densely granular and darkly staining, the ripe cell greatly swollen, with paler contents (Text-fig. 6 A, B). These cells occur frequently in groups, opening by a common pore, and the extrusion of the sticky secretion can readily be observed in life. They occur thickly all over the caecal valve, columellar ridge, and adjacent main mucous collecting ridges, and line the caecal wall in this region. They are confined, in the leaflets, to the free edges of the largest leaflets (Text-fig. 5A), particularly towards the collecting ridges, although scattered isolated cells of this type appear occasionally on secondary leaflet ridges, close to the mergence of the leaflet grooves with the collecting grooves. Gariaeff (1915, p. 51, Pl. VI, figs. 70, 71) figures both types of cell; he describes the cells of the secondary leaflet ridges as mucous, but not those of the leaflet edges, despite their reactions with Mallory, as he failed to stain them with mucicarmine. Their staining with Mallory and Prenant as well as their appearance and behaviour leave little doubt as to their nature.

Thus, with the exception of the hepato-pancreatic groove, the caecum's whole activity is concerned with the final stages of a complete extracellular digestive breakdown and with the absorption of the results thereof. Absorption takes place principally in the sac, facilitated by the absence of mucus and the resulting stirring action of the sac cilia (p. 22). It may be noted that the blood-supply, though well developed, is not of that great richness to be anticipated in an organ of absorption of this type, and the possibility cannot be excluded that some of the absorbed products pass through the thin layer of the caecal wall directly into the fluids of the perivisceral coelom.

The Intestine and Rectum

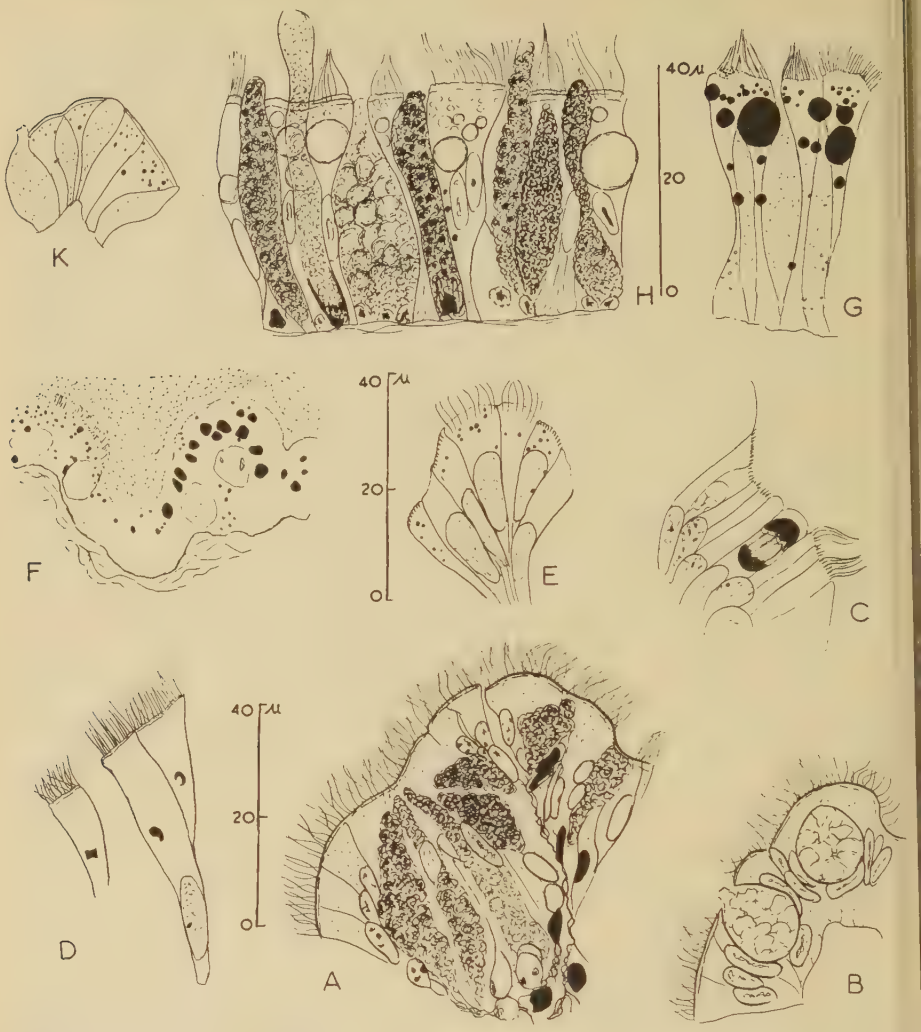
The *intestine* of *Loligo* is a tapering tube, which passes between the two halves of the pancreas and along the ventral surface of the liver, where it leads

into the *rectum*, which opens just behind the funnel (Text-figs. 1, 2). The main mucous groove from the caecum (p. 18) can be distinguished for a short distance along the ventral wall of the intestine (Text-fig. 4B), whose whole inner wall is corrugated with a series of longitudinal folds which, even in the distended organ, are traceable as ridges of connective tissue and longitudinal muscle-fibres. The ridges of the main mucous groove, called by Gariaeff (1915) the 'typhlosole', are further heightened close to the caeco-intestinal opening by underlying ridges of cartilage, similar to the cartilage of the caecal valve (p. 25). The anus is a slit-like opening in a small muscular 'rectal bulb' and is guarded by two 'anal leaflets' (Text-fig. 2). The duct from the ink-sac opens close to the bulb, and the rectum can be closed on each side of this opening. The delimitation of the intestine and rectum is here considered to be a histological one (see p. 29). A substantial outer layer of circular and a thinner, inner layer of longitudinal muscles is present, similar to that of the oesophagus, except that the layer of longitudinal muscles is continuous, and the blood-supply, as might be expected with an absorbing epithelium, much richer, and conspicuous in both the inner and outer layers of connective tissue.

Waves of contraction are frequently to be seen passing along the intestine, continuing for some time after death. In late stages of digestion the intestine may be full of fluid resembling that in the caecum.

The whole of the intestine is lined with a ciliated and mucous epithelium (Text-fig. 6 G, H). As in the ciliated organ, variations in the height of the epithelium accentuate and help to form ridges and grooves, but the mean height here varies greatly with the degree of contraction of the circular muscles; it is also greatest in the widest part of the intestine, near the caeco-intestinal opening, and diminishes steadily towards the rectum. The ciliated cells have long cilia with basal granules, and indications of short, parallel rootlets. The cells have a well-marked cell-border, staining brilliantly with eosin in Prenant-Hollande, differing in this from the cells of either the ciliated organ or caecal sac. The oval nucleus, with one or more nucleoli, is more or less in the middle of the cell, unless displaced by an adjacent ripe mucous cell. The basal portion, often tapering, is markedly fibrillar; the fibrillae, as in the oesophageal and gastric cells, are united with the basement membrane, itself intimately connected with the underlying connective tissue. In late stages of digestion the distal half of the ciliated cell is packed with absorbed fat-droplets. Single basiphil granules are found irregularly in the distal half of these cells. Scattered thickly among the ciliated cells are mucus-secreting goblet cells, which, in their method of secretion and staining, resemble those of the collecting ridges of the ciliated organ, but which always open individually to the intestine. Gariaeff (1915, p. 52) also regarded these cells as mucus-secreting.

Copious supplies of mucus are produced by these cells, and can continue for many hours after death: for an hour or so even in small fragments of intestine. This mucus protects the intestine from the sharp faecal residue, as the gastric cuticle protects the stomach (p. 14).



TEXT-FIG. 6. A and B. T.S. through two leaflet-edges (cf. Text-fig. 5) to show the vesicular type of mucus-cell: A, ripening cells, fixed Helly, stained Prenant-Hollande; B, ripe cells, fixed Zenker, stained Mallory.

C. Mitosis in a ciliated cell of a secondary leaflet groove; D. 'Golgi-like' bodies in leaflet cells, cf. Plate II; C and D fixed Helly, stained iron haematoxylin.

E. Fat-droplets in apical cells of a leaflet-ridge; F. Fat-absorption in the caecal sac; G, H. Fat-absorption and mucus-secretion in the proximal part of the intestine; K. The same in the 'hyaline border' region of the rectum. E, F, G, K, fixed Flemming-without-acetic, unstained; all taken from the same animal. H, fixed Helly, stained Prenant-Hollande. In H the fat-droplets are represented by empty vacuoles.

The currents formed by the ciliated cells are all directed towards the anus. Their function would seem to be like that of the caecal sac: to renew the layer of assimilable food next the absorbing surface. There is no indication of cilia and mucus working together to collect solid particles; indeed the absorbable

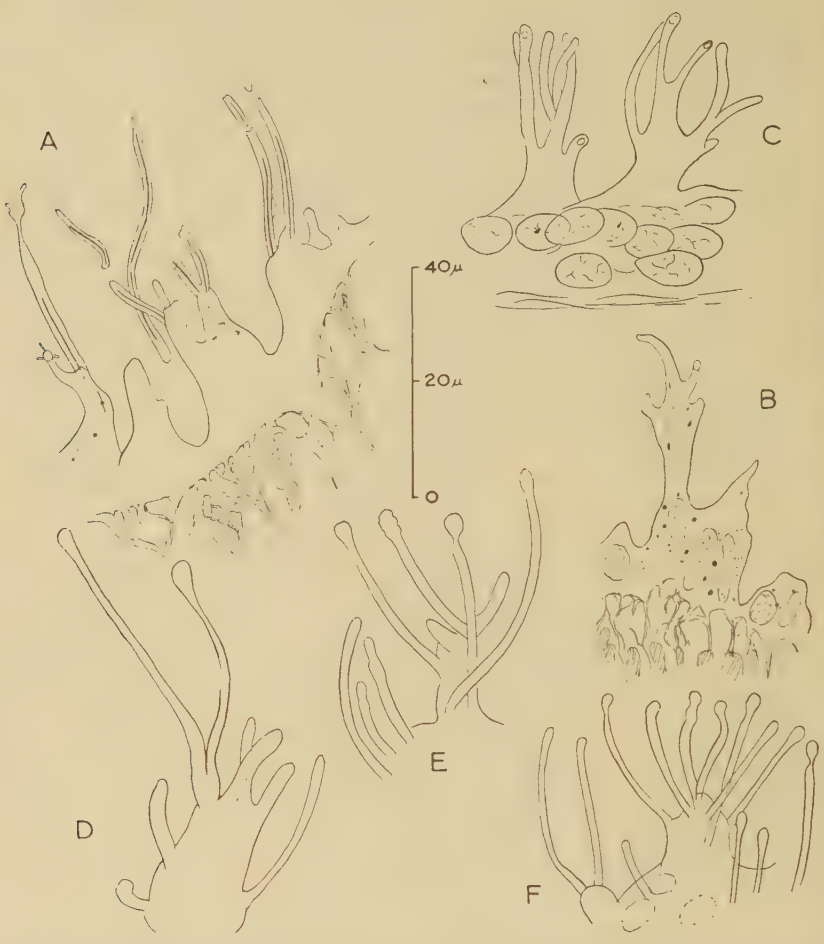
fluid passed to the intestine from the caecum has already been cleared of solid particles which, passed along the intestine in the string of the main mucous groove (p. 23), do not interfere with intestinal absorption.

It seems probable, from the mixture of completely and partially digested residue often found in the stomach, that defaecation does not take place till the meal is ended, so that intestinal absorption is also unimpeded by the passage of large faecal masses. It is curious that intestinal absorption would seem to be just as active and efficient as that of the caecal sac, despite the large number of mucous cells. It is possible that the activity of these cells is discontinuous: the copious supply described above in an excised portion of intestine was associated with repeated strokings of the surface by a needle to free the epithelium of mucus, and the mechanical stimulation of the contact of gastric faeces may be the necessary stimulus for mucus-secretion.

The junction of intestine and rectum may be regarded as marked by the replacement of the ciliated cells by lower cells with a hyaline border (Text-fig. 6K). These contain fine fat-droplets, and are also interspersed with mucous goblet cells. This part of the rectum is short: 2–3 mm. in a fully grown specimen.

The last part of the rectum, including the anal bulb and the opening of the ink-duct, is lined with most remarkable cells forming a cubical epithelium whose cells can put out long retractile processes. The longitudinal grooves of the intestine break up in this region in a fine network, so that the epithelial cells form cushions, from which these processes protrude in bunches, sometimes individually, when the processes can be traced, each one to an individual cell, and may reach a length of over 50μ, sometimes as blunt protrusions from a single mass (Text-fig. 7). A vacuole may be visible in the tip, which is often slightly swollen, especially in fully extended processes, and one or more fine processes may in turn be seen projecting from the terminal knob. Each process has an 'axial filament', faintly visible in unstained sections fixed with Flemming-without-acetic, and staining black with iron haematoxylin after the same fixative. When a process is cut off short in the section, the axial filament may be seen, projecting like a stiff rod from the cut end (Text-fig. 7A). The same fixative shows scattered fine fat-droplets in the cells, and occasionally in the processes. Slow changes of form have been seen in the adult living cell, and in newly hatched larvae the processes are well developed, and wave to and fro in currents of water drawn in and out of the rectum by contractions of the intestine: the appearance in sections of adult material strongly suggests the same flexibility. The cells are described both by Williams for *L. pealeii* (1909, p. 41, Text-fig. 13), who observed them in larva and adult, and by Gariaeff (1915, p. 53, Pl. VII, fig. 93); their accounts agree on the whole with the present description. Williams had observed water being pumped in and out of the larval rectum, and had found that carmine, carried in on a rectal current, was retained in the rectum, he suggested by the action of these processes, when the water was expelled. He also found small bodies, corresponding in size and distribution to the fat-droplets here shown in Text-fig. 7, in the adult processes, and deduced a 'screening' action in the larvae and phagocytosis in

the adult. Gariaeff regarded the terminal vacuoles as secretory, and claime to have found collapsed processes from which the secretion had been dis charged (his figures, however, suggest that this appearance may have been du



TEXT-FIG. 7. Rectal cells of *Loligo*. A and B from T.S. of rectum of the young specimen of *L. vulgaris*, fixed Flemming-without-acetic, whose caecal and intestinal cells are drawn in Fig. 6 A, stained iron haematoxylin, the nuclei invisible; B, unstained. Both show the radiating connective-tissue fibres attached to the epithelium, interspersed with bundles of longitudinal muscle fibres (cross hatched). C from L.S. of rectum of half-grown *L. vulgaris*, fixed Bouin-Duboscq (with ammonium sulphide), stained carmalum. D, E, F, quick sketches of living rectal cells of full-grown *L. forbesii*, dead 1–2 hours.

to imperfect fixation). For him, the cells secrete a coagulent which prevents ink from penetrating into the intestine.

Neither explanation is entirely satisfactory.

The general appearance of the cells does indeed suggest some phagocytic activity, yet it seems meaningless to find phagocytosis in the last few milli-

metres of an alimentary canal along which the food is driven by active peristalsis. Moreover, the solid food which passes the region where these cells are situated is in the form either of relatively large and quite indigestible fragments from the stomach, or of the mucus-string from the ciliated organ of the caecum. It is true (p. 24) that some nutritive particles are entangled on this string, but, were these to be retrieved, such action might be anticipated during the string's relatively slow progress along the intestine, and of this there is no sign. The nutritive liquid, which is passed on to the intestine for absorption, has already been cleared in the caecum by the ciliated organ of all particles suitable for phagocytosis.

If the function of the rectal cells be unrelated to the digestive mechanism, then Gariaeff's association with ink-secretion may be considered. Here again the violence with which the ink is ejected makes it difficult to imagine a part played by the rectal cells. The coagulation of ink which he observed in the intestine may well have been due to the mucus freely secreted by the intestine itself, if not to that which usually appears in a cloud of ejected ink. The extreme dissimilarity between the rectal cells and any known type of secreting cell makes the idea of a secretory activity difficult to accept.

A third possibility is that the reversed peristalsis by which water is drawn into the larval intestine occurs also in the adult, that water is absorbed in the intestine, and that the processes are associated with this activity. It is conceivable that they have a sensory function, testing the indrawn water, and might be associated in this with the anal leaflets, to which no function has been ascribed. It must be admitted, however, that the rectal cells are as unlike any known type of sensory as of secreting cell. It is of interest to note that cells of very similar proportions at the junction of the mid-gut and rectum in *Rhodnius* are described by Wigglesworth (1931, pp. 432, 437, figs. 3, 5), who found them swollen and active during excretion. These cells, however, are 200μ in length, three times the length of the rectal cells of *Loligo*, and, lying side by side, fill the lumen of the rectum completely, so that the whole scale of activity is very different from that of the 60μ processes fringing the last few millimetres of the intestine of a 300 mm. long *Loligo*.

The Mid-gut Gland

With the possible exception of the ciliated leaflets of the caecum (p. 25), there is no evidence to suggest that the alimentary canal or the glands of the fore-gut contribute any digestive enzymes, and we must seek in the mid-gut gland all the enzymes necessary for complete extracellular digestion.

The mid-gut gland consists, as in all dibranchiate cephalopods, of two very unequal parts (Text-figs. 1, 2), for which, for convenience, the names given by early writers of 'liver' and 'pancreas' are retained. The gland arises in development as a pair of finger-like diverticula from the mid-gut. The major, anterior part of each diverticulum becomes the liver, the lesser, posterior part, the hepatic duct and pancreas (Portmann and Bidder, 1928, Figs. 2B, 15B). The duct is non-glandular where it leaves the liver; as it nears the mid-gut, the

walls become elaborated into the spongy pancreas; when the mid-gut becomes differentiated into stomach and caecum the latter, as we have seen, contains the hepato-pancreatic opening. The two glands are thus connected in series and their structural plan is the same: a system of ramifying tubules, opening into a wide central lumen on each side. The tubules are each surrounded by a blood-system which Vigelius (1881, 1883) has shown to be truly capillary. Despite this common plan, the two glands are very different in size, appearance, texture, histology, and mode of action.

The *liver* is a cigar-shaped organ, occupying all the anterior part of the body (Text-fig. 1A). It lies between the retractor muscles of the funnel and abuts against the posterior face of the skull, partly surrounding the poison gland; it is covered by a delicate, transparent muscular sheath. The paired origin of the gland can be traced by the general arrangement of its tubules, and by the passage of the oesophagus and aorta through it (Text-figs. 1, 2). It varies from pale sand-colour to a rich tan-brown, and is of very soft, almost liquid texture. The lumina of the smaller tubules are often obliterated by the swollen ends of the ripe liver cells. The delicate capillary system is generally empty in preserved material.

The non-glandular paired hepatic ducts leave the liver on each side of the oesophagus and aorta. Where the duct leaves the liver a sphincter is formed by a circular layer of muscle-fibres, some of them striated (p. 13), and with a few fibres radially and obliquely arranged.

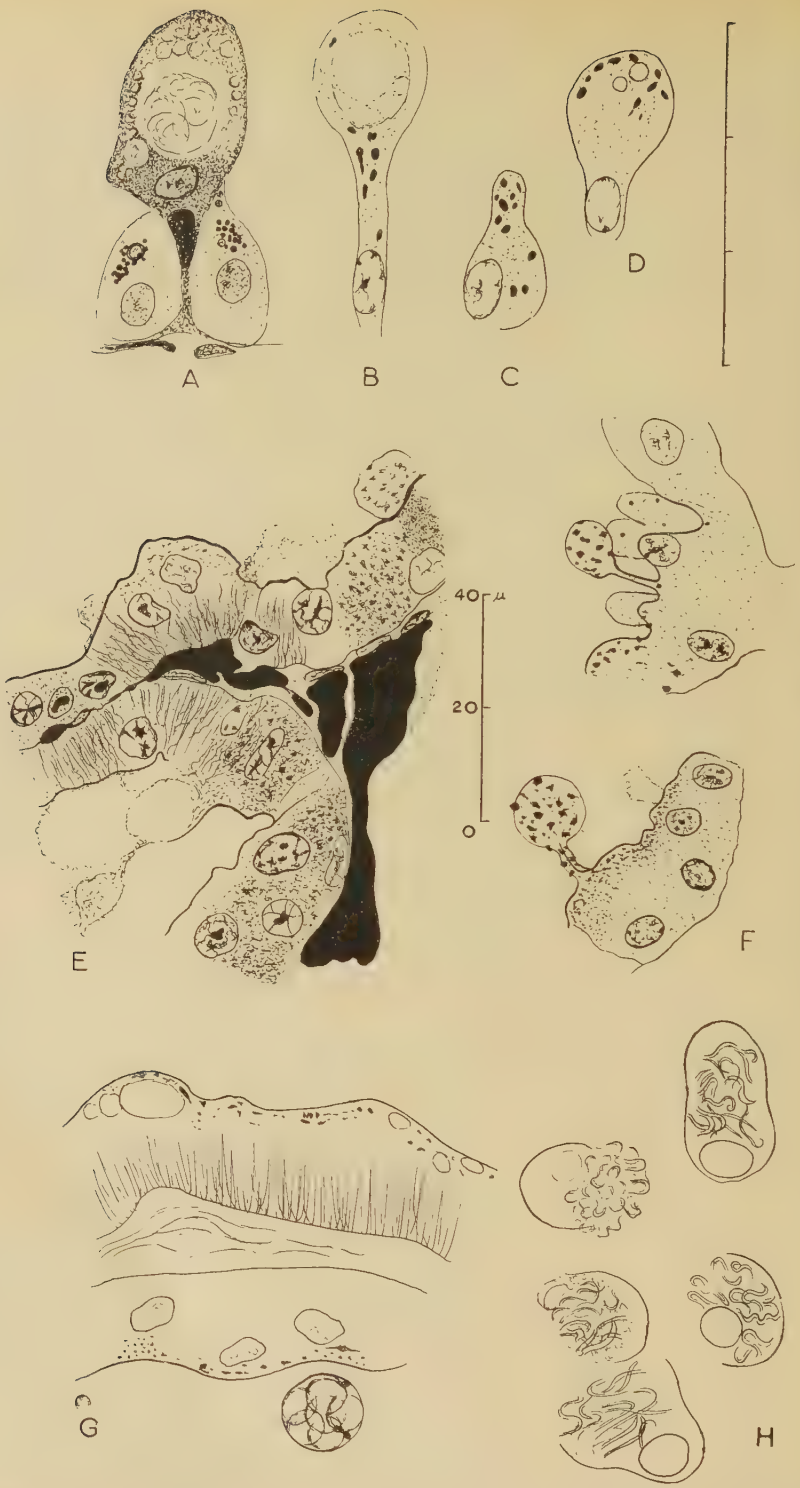
The *pancreas* is a small, wedge-shaped organ, whose volume is about one-tenth that of the liver. It lies completely within the kidney sacs and is covered by a single layer of renal epithelium. It is further in intimate contact with the adjacent kidney tissue, and its blood-supply drains into a pair of large vessels, opening directly into the venae cavae of each side and, like them, covered with renal tissue. The cavity of each hepatic duct expands within the pancreas into a wide lumen, with which the large tubules of the spongy walls communicate freely. The pancreas is sometimes colourless and translucent, sometimes creamy-white and opaque, and is elastic and tough, so that a teased preparation is difficult to make and a smear impossible. This is due to the blood-system, which is reinforced, even in the fine capillaries, with connective-tissue fibres, and is conspicuous in teased or macerated material, or in sections, in which it is usually distended with brightly staining blood. The tubules are wide and their epithelium low (Text-fig. 4B). The opening to the caecum is protected by the hepato-pancreatic fold (Text-figs. 3, 4), but there is no mechanism to prevent the flow of secretion from the gland.

The secreting cells of the two organs show an equally great contrast. The ripe club-shaped liver cells alternate with triangular basal formative stages (Text-fig. 8A). Two elements of secretion are formed in the liver cell, but they mix in the hepatic tubules, and are liberated together. One appears in the basal cells as a single ball which swells when it reaches the swollen distal end of the ripe cell into one large or a group of smaller vacuoles; the second forms a cluster of granules, which also appear first in the basal cells, and follow the

ball into the distal part of the cell, where they usually form an outer layer surrounding the central large vacuole or vacuoles, and then swell to form minute vacuoles or droplets. The large ball is sometimes unstained and yellowish, sometimes lightly basiphil in the unripe cell; the contents of the vacuole into which it ripens are strongly acidophil (green with Prenant-Hollande, blue with Mallory), colourless in the living cell, and staining lightly orange with neutral red as the cell dies, when a small concretion may appear. (The liver cell undergoes rapid alteration after the death of the animal, and only those observations made within a few moments of decapitation can be regarded as related to a normal cell.) The ripe vacuoles sometimes contain droplets of unsaturated lipoid material (giving secondary blackening only with osmium tetroxide, orange with Sudan III, blue with nile blue). This fatty material is present in very varying quantities, sometimes almost filling the vacuoles, sometimes absent, but its occurrence has not been related to the digestive process, since it may be absent in feeding, present in hungering animals. The granules are more basiphil than the vacuoles, staining densely black with iron haematoxylin until they have reached their distal position, where, as they swell into droplets, they become more acidophil (staining strongly with eosin in Prenant-Hollande), while the black colour is first reduced to a shell and then disappears. In the living cell the ripe droplets stain dark red with neutral red but disappear in a matter of seconds as the cell dies. While these granules are passing through the middle part of the cell, they give a strong reaction for iron (p. 6), which is also shown by the shell of the ripening droplet. Two nuclei are often to be found in the middle portion of the cell (Text-fig. 8A); when secretion takes place the whole swollen end of the cell is cast off, often including one of these nuclei; mitotic figures have been seen in the basal regenerating cells.

Two cycles of secretion may be identified. In 'unhurried' secretion the ripe cell-end contains both vacuoles and droplets, fully matured, before it is cast off. In 'short-cut' secretion the cell-ends are cast off before the granules have reached them, and contain the large vacuoles only; a second secretion follows of cell-ends containing granules only (Text-fig. 8 C, D). Secretion may occur before the granules swell into droplets, which then takes place in the lumen of the gland. Short-cut secretion is associated with outbursts of secretion during the meal; unhurried secretion also takes place during the meal, but is characteristic of the gland of a hungering animal, or of one 6 hours after the capture of food, when the whole digestive process is normally completed.

The cells are continuously active; the proportion of basal to club-shaped cells varies, but some basal cells are to be found at all stages of feeding and hunger. The secretion is retained in the gland between meals, and only appears in the caecum about 30 minutes after the capture of food. Storage of secretion has been traced in the liver of living animals which had been fed with nile blue: the liver remained darkly blue for as much as 48 hours in an unfed animal, but lost colour after feeding. The secretion is partly stored in the swollen distal ends of the cells, which then completely fill the smaller



TEXT-FIG. 8. Cells of the liver (A–D) and pancreas (E–H); A, F, fixed Helly, B, C, D, E, fixed Zenker, all stained Prenant-Hollande; G, H from living cells. Scale the same throughout.

tubules, partly in the central lumina, where vacuoles, granules, and discarded cytoplasm all break down to a homogeneous liquid.

The low pancreatic epithelium (Text-fig. 8 E–H) has a well-marked basiphil cell-border, and consists of sub-cubical cells, whose cell-boundaries are usually invisible in section but which are readily separated in teased or crushed material. The broadly oval nuclei (rather larger than those of the liver cells) are usually apical in position; the cytoplasm is conspicuously fibrillar. The fibrillae are easily seen in living tissue, and are visible in isolated living cells as highly refringent threads, bent by the contraction of the free cell into shapes suggesting some elasticity (Text-fig. 8H). As the cell dies the fibrillae appear to break into granules which stain with Höchst's Janus green B. Fibrillae and granules are visible both in preparations made by Regaud's method for mitochondria, and those following Zenker fixation, in which mitochondrial matter had presumably been dissolved away. Vacuoles of secretion appear between the fibrillar cytoplasm and the cell-border, often appearing in preserved material as solid spheres, but in life as vacuoles filled with droplets or lesser vacuoles (Text-fig. 8). Within and among the vacuoles are scattered granules, staining blue with nile blue, red with neutral red, and black, in preserved material, with iron haematoxylin; the whole gland tends to be basiphil in staining. Secretion occurs rhythmically and well-marked resting periods intervene apparently throughout the gland; this may be related to the macroscopical variations in appearance. The rhythm of secretion has not been related to the meal. No mitotic figures have been seen, but degenerate nuclei are frequently found in the lumina of the tubules and within the epithelium. No lipoid material has been found beyond the small granules already mentioned. In the iron saccharate experiments the pancreas was, unfortunately, very badly fixed; no trace of iron was ever found in the gland.

There is but little knowledge of the exact nature of the hepato-pancreatic secretion. Sellier (1910) gives an account of the proteolytic enzymes of the liver and pancreas taken together; Williams (1909, p. 40) states that the extract of the liver is proteolytic, that of the pancreas proteolytic, amylolytic, and lipolytic. Dr. L. E. Bayliss, who kindly made a brief examination of the liver and pancreas of several specimens of *L. forbesii* at Plymouth in 1933, found both proteolytic and lipolytic enzymes in both liver and pancreas. The lipase showed a pH optimum of between 6·6 and 6·8; the protease appeared to have an optimum in the region of 7·2. Dr. Bayliss found the pH of the caecal fluid to be between 5·6 and 5·8. Some earlier observations by the present writer, made on freshly dead trawl-material, gave the pH of the stomach 6·2; caecum 5·6–5·8; pancreas 6·0; liver 6·0. The figures obtained for the enzyme activity of the liver suggested considerable individual variation in the extract, relatable to the physiological state of the gland. The data for the pancreatic extract are of doubtful value, since, as the pancreas is but a glandular elaboration of the hepatic duct, the main pancreatic tubules are liable to be filled with hepatic secretion.

Romijn (1935, pp. 421, 426) found an amylase, but no maltase or saccharase in the 'Magensaft': it is not clear whether this is, in fact, stomach contents caecal contents, or a mixture (see below). He found a pH optimum of 6·26 for starch digestion and of 5·96 for digestion of glycogen.

None of these observations throws light on the particular role of either liver or pancreas in the digestive process, particularly in gastric as distinct from caecal secretion. Do both glands contribute to each phase, or is one responsible for gastric, the other for caecal digestion?

It is clear that the brown colour which appears in the caecum during meals (p. 9) must be hepatic in origin, and, since only one secretion is produced by the liver, the pancreas must be responsible for the colourless fluid which fills the caecum during hunger (p. 10). The brown hepatic colour has not been detected in the stomach, so that it is tempting to infer that the gastric juice is also pancreatic in origin and that gastric digestion begins under the action of the caecal 'hunger fluid', supplemented, once caecal digestion has begun, by the flow of pancreatic secretion directed to the stomach along the hepato-pancreatic groove, or, more properly, the pancreatic groove. This would be possible (1) if the hepatic sphincter (p. 32) remained shut while the hepato-pancreatic fold closed on to the columellar ridge, thus making the hepato-pancreatic groove a closed tube leading to the stomach (p. 16), and (2) if the sphincter relaxed only when relaxation of the hepato-pancreatic fold gave the hepatic secretion access to the caecum. If the pancreatic extract of *Loligo* be activated by the secretion of the caecal lining (cf. p. 25), as Romijn (1935, p. 421) found to occur in *Sepia officinalis*, then we might suppose that enough of the activator passed into the stomach with the first drive of digestive juice from the caecum. It is, however, possible that the activating system, if present in *Loligo*, acts on hepatic, not pancreatic secretion: there is at present no justification for generalizing from the Sepioidea to the Teuthoidea, even though Romijn found similarities in the starch-splitting enzymes—particularly as it is not clear whence his 'Magensaft' was derived. It seems improbable that, with the large caecum available, juice should be taken from the stomach of *Loligo*, either full of food or contracted and empty; while the hungering stomach of *Sepia* is often distended with fluid. Romijn may thus have compared the stomach juice of *Sepia* with the caecal juice of *Loligo*. Preliminary investigations by the present writer suggest substantial differences between the digestive mechanisms of the two genera—including the use by *Sepia* of the stomach, not the caecum, as a store of 'hunger-secretion'. Caecal activation of hepatic secretion would make possible storage of secretion in the gland.

One of the most striking differences between the liver and pancreas is that the pancreatic secretion is liberated as it is formed (p. 32), accumulating in the caecum between meals and passing to the stomach during gastric digestion, while the hepatic secretion is only liberated during digestion. This control is effected by the hepatic sphincters, whose striated fibres (pp. 13, 32) would effect a rapid closing action; its non-striated fibres control the prolonged closure of the sphincter between meals. The duct is supplied with nerves from

the splanchnic ganglion (Alexandrowicz, 1928), so that nervous co-ordination is possible between the sphincter and the hepato-pancreatic fold, in the manner suggested above. It is less clear what stimulus initiates relaxation of the sphincter and liberation of secretion during digestion. This has been initiated by decapitation or by the subsequent handling and dissection in two animals at very different stages of digestion. Both had been fed with nile blue, one $2\frac{1}{4}$ hours, the other 17 hours before decapitation. In each the liver when opened was clearly stained; in each the stain had appeared in the caecum and completely vanished from the liver 35–40 minutes after decapitation: despite the very different physiological states of the two animals decapitation in each case stimulated the liberation of hepatic secretion. Further evidence of nervous control was derived from one animal which was captured with the stomach distended with half-digested food, but with no sign of hepatic secretion in the caecum, which only appeared 4 hours after capture, after which digestion was completed at less than the normal speed (cf. p. 10). The shock of capture would here appear to have had an inhibiting effect on the liberation of hepatic secretion.

These observations, while demonstrating clearly the nervous control of the sphincter, throw no light on the immediate stimulus which normally induces it to open.

It may be noted that Sellier (1907, 1910), who investigated a colourless caecal juice, was thus probably examining pancreatic secretion. It is curious that he found no difference between the caecal fluid in hunger or digestion, but, as he describes no difference in colour or turbidity, he probably never examined an animal at the height of hepatic and caecal activity. The present writer never observed more than the faintest hepatic tint in the caecum of trawl-material, in which capture may regularly inhibit liberation of hepatic secretion.

It is clear that careful examination of liver and pancreas extracts is needed, as well as of caecal and gastric juice and caecal extract, all at known stages of digestion.

It seems, however, reasonable to postulate that the pancreas rhythmically liberates the enzymes necessary for the preliminary gastric digestion, while the liver continuously produces those necessary for the final caecal digestion, but only liberates its secretion while digestion is taking place. These two activities do not in themselves account for the differences between the two glands, either in bulk or histology, and it is clear that each must have some other activity.

It is natural to look for an absorptive function in a molluscan mid-gut gland, but, in fact, the evidence is against absorption by either liver or pancreas. The iron experiments were inconclusive on this point (p. 6), but the histology of the liver definitely excludes hepatic absorption: every type of cell can be referred to a cycle of secretion and can be found in an animal which has hungered for many hours (p. 33); further, the only free surface offered by the hepatic epithelium is that of the ripe cell-end, about to be cast off in secretion.

The evidence against pancreatic absorption is more circumstantial. The well-guarded opening makes entry of food into the gland improbable (Text-figs. 3, 4). The almost complete absence of lipoid matter from the pancreas (p. 35) is also strong evidence, since unabsorbed fat is still present when the digested food passes on from the caecum to the intestine (Text-fig. 6), nor does the low pancreatic epithelium with its characteristic apical nuclei resemble most known absorbing tissues. There is no good reason, in fact, for postulating pancreatic absorption and several against doing so. The differences between the two glands must be due to other causes.

The great bulk of the liver must be related to storage of reserves: no other tissue of the body suggests this function. The reserves may be of a protein nature, as suggested by the strongly basiphil staining of the permanent, basal part of the epithelium (Sellier found a high protein content in the liver which he held to be due to hepatic absorption), or may be glycogen, detected by Chaigne (1934) in many tissues, including the liver, of other cephalopods. The presence of iron and the curious and irregular production and liberation of lipoid material, an activity calling for further investigation, also differentiate the liver sharply from the pancreas. An excretory function was attributed to the liver by Cuénot (1907, p. 34), on the basis of the appearance of injected Jodgrün and Echtroth in cells of the liver, as well as in the kidney, 24 hours after injection.

What function, other than that of enzyme secretion, is performed in the pancreas is at present obscure. It may be related to the long 'resting' phase between secretion-production, to the apical nuclei, the well-developed blood-supply, wide tubules, and supporting connective-tissue system, although this last may be only to give strength against the pressure of the hepatic secretion passing through the gland. The wide tubules must result in free contact between the hepatic secretion and the pancreatic cells of all but the smallest tubules, which suggests the possibility of some interaction between the two. This already exists in the larva (Portmann and Bidder, 1928) in which the developing liver surrounds the internal yolk-sac, and the yolk is taken up by the liver cells, passed down to the pancreas, by which it is absorbed into the blood-stream. The intimate connexion between the pancreas and the kidney must also have some functional significance and may be related to the great volume of secretion produced by the pancreas. On the other hand, the histology suggests that the second pancreatic activity may be directed towards the blood-stream, and the possibility of internal secretion cannot be excluded. The difficulties in obtaining pure pancreatic secretion, or of making any experiment involving operation on so highly strung an organism as *Loligo* indicates that the answer to this problem is to be sought by the methods of histochemistry.

Conclusion

The Meal

The digestive mechanism of *Loligo* and *Alloteuthis* which has been outlined in these pages may now thus be summarized:

Food, killed by the action of the poison glands, is bitten into pieces by the jaws (p. 9) and swallowed with the help of the radula and probably of lubricant from the buccal gland (p. 12). In the stomach it encounters colourless pancreatic secretion, which has been stored in the caecum (p. 36). This is driven into the stomach by strong contractions of the caecal sac, the caecal valve being held open by the distension of the anterior part of the caecum (p. 18). Under the influence of the pancreatic enzymes, and through the churning action of the stomach (p. 9), the food is broken down to particulate or liquid form. About half an hour after the capture of food the first consignment of partially digested food is passed on to the caecum (p. 10). When this happens the muscles of the caecal valve, hepato-pancreatic fold, and hepatic sphincter relax so that the caecal valve, acting as a simple valve, allows food to enter the caecum where it is met by an outpouring of hepatic secretion. The caecal valve and hepato-pancreatic fold then close (p. 18), so that the contents of the stomach and caecum are isolated from one another and muscular movements of either will not mix their contents. The hepatic sphincter also closes, so that pure pancreatic secretion passes to the stomach where preliminary digestion proceeds, while, in the caecum, the muscles and cilia of the sac combine to keep the contents gently moving (p. 22) and digestion is completed under the action of hepatic secretion (p. 36). At intervals, further consignments of partially digested food and hepatic secretion meet in the caecum, where the ciliated organ is continuously removing solid particles into the mucous groove of the intestine (p. 22), while absorption begins in the cells lining the caecal sac (p. 25). Gastric digestion lasts (on an average) 1½–2 hours; caecal digestion and absorption are completed after about 4 hours (p. 10). While absorption is still proceeding in the caecum, the caecal valve is opened (p. 19) and some of the caecal contents are driven into the intestine where absorption begins (while caecal absorption is still active) and continues after caecal absorption has ended (p. 27). At an unknown interval after the end of intestinal absorption, sometimes only after a second meal has been captured, digested, and absorbed, the indigestible residue from the stomach is passed out, well wrapped in protective mucus secreted by the intestinal lining (p. 27). The caecal valve, held shut by the muscle in its rim (p. 18), prevents the faeces from entering the caecum.

When digestion is at an end the hepatic sphincter remains closed, the hepato-pancreatic fold and caecal valve relaxed, so that hepatic secretion accumulates in the liver, pancreatic in the caecum (p. 36), while simple valve action of the caecal valve retains the pancreatic secretion within the caecum.

It may be noted that intestinal absorption is probably especially important at sexual maturity. At this time the gonads may occupy almost the whole space generally occupied by the caecal sac. The appendix, even when fully distended, could not compensate fully for the loss either of absorbing surface or of cubic content, and the intestine is probably important at such times, not only for absorption, but for holding unabsorbed food.

Discussion

The most striking facts of digestion in *Loligo* are the speed and completeness of the digestive process, and the complexity of the mechanism by which this is achieved.

The whole of the structure may be interpreted in terms of speed and efficiency. Separation of the mid-gut into stomach and caecum makes possible separation of the digestive process into two phases, a preliminary phase in the stomach, a final in the caecum, which can go on independently and simultaneously under the action of their appropriate enzymes. The division of the mid-gut into two parts, and the complex structure of the hepato-pancreatic fold are closely associated with this division of the digestive process into two phases. The speed of the preliminary gastric digestion is probably due partly to the character of the pancreatic secretion, but largely to the violent churning action of the stomach itself.

Exclusion from the caecum of large masses of undigested food or indigestible residue must greatly facilitate the final stages of both digestion and absorption of the resulting soluble foodstuffs. The development of a large blind sac as an organ of absorption is made possible by the presence of the ciliated organ: this (1) prevents the accumulation of solid particles, which would otherwise increasingly clog and obstruct the lumen, as only the fluid is absorbed, and (2) frees the absorbing epithelium entirely from solid particles for the greater part of the absorptive phase. The ciliated organ has its parallel, and almost certainly its homologue, in the ciliary mechanisms of the primitive gastropod and lamellibranch mid-gut (Graham, 1949). In these groups, however, as frequently in invertebrates, secretion and absorption take place side by side, or successively in the same cell, in a single digestive diverticulum of the mid-gut. By developing a second diverticulum for the absorption of soluble food, completely free from enzyme secretion, *Loligo* is enabled to use its molluscan inheritance to form a digestive mechanism of startling efficiency.

The whole digestive mechanism is admirably adapted to the life of a ceaselessly swimming animal which is a predator on shoaling animals, and itself the prey of active predators. The segregation of gastric and caecal digestion are particularly well fitted to allow of continuous feeding when a shoal is encountered, or of feeding whenever food is available without interfering with the digestion of an earlier meal. The rapid digestive process (4–6 hours, in contrast to 40–60 hours recorded by Dawes (1930, p. 93) for the plaice)) reduces to minimal duration loss of activity due to the inertia of repletion. It may be noted that the habit of storing pancreatic secretion in the caecum results in that organ being of more or less constant bulk in feeding and hunger, which should further diminish the effect of feeding on swimming efficiency.

The economy of the digestive process enables the fullest use to be made of any food that is captured. The only assimilable food which is lost is the small amount of fat-droplets and food particles carried out in the mucus-string

from the caecum: compared with the large quantities of utilizable food passed out with the faeces of other carnivorous animals, this wastage may be regarded as negligible.

Especially noteworthy is the delicate interplay of the muscular action of the stomach, caecal sac, oesophageal, intestinal, and hepatic sphincters, and hepato-pancreatic fold and caecal valve. The position of the splanchnic ganglion at the junction of oesophagus and stomach must greatly facilitate the accuracy of this control. It would be of great interest to know exactly what stimuli produce the various conditions allowing food to pass from the stomach to the caecum or from the caecum to the intestine, enzymes from the caecum or pancreas to the stomach or from the liver to the caecum, or faeces from the stomach to the intestine.

The histology of the alimentary canal offers many fascinating problems for further study, not least among which are the pancreatic and rectal cells.

In conclusion, it must be stressed that the picture of the digestive mechanism set forth in these pages is only in part applicable to other Cephalopoda. Even within the Loliginidae, Williams's account, and the present writer's unpublished observation on *L. pealeii*, show that the closing apparatus of the caecum differs markedly within that family, and greater differences are to be found when the comparison is extended to the Cephalopoda as a whole. While the basic plan of structure described for the ciliated organ of the caecum of *Loligo* has been found by the present writer in every cephalopod examined, other features, such as the degree of coiling of the spiral, presence or absence of the sac, and the nature of the closing mechanism of the caecum, show a wide range of variation. Again, while in all described Dibranchiata the mid-gut gland may be divided into 'liver' and 'pancreas', the histology of the liver varies considerably, and the pancreas shows even more marked variation in its relation to the hepatic duct on the one hand and the kidney on the other (Vigelius, 1881, 1883; Castaldi and Musio, 1928). The duration of digestion is also very variable, 18 hours in *Octopus* (Falloise, 1906), 12 in *Sepia* (Gariaeff, 1915): two or three times the length of the digestive process in *Loligo*.

Falloise found in *Octopus*, as the present writer found in *Loligo*, that food did not enter the mid-gut gland, but Gariaeff (1915, p. 90) claimed to have traced the entry of particulate food into the liver of both *Octopus* and *Sepia*, which the present writer, repeating Gariaeff's experiments on *Sepia officinalis*, is at present unable to confirm.

Some of these apparent discrepancies may prove to be due to erroneous observation, but some to the profound difference between the ways of life of the bottom-dwelling, lurking *Octopus* and partially bottom-dwelling *Sepia*, and the perpetually swimming pelagic *Loligo*. Until more information is available it is clearly dangerous to combine, as has too often been done, one observation from *Octopus* with another from *Sepia* under the generalization 'In the Cephalopoda'.

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REFERENCES

ALEXANDROWICZ, J. S., 1928. Arch. Zool. exp. gén., **67,** 69.
BIGELOW, H. B., 1928. Bull. U.S. Bureau Fish., **40,** 1.
BOTTAZZI, F., 1916. Publ. Staz. Zool. Napoli, **1,** 59.
—— and VALENTINI, V., 1924. Arch. di Sc. Biol., **6,** 153.
BOURQELOT, EM., 1882. Arch. Zool. exp. gén., Sér. 1, **10,** 385.
—— 1885. Ibid., Sér. 2, **3,** 1.
CASTALDI, L., and MUSIO, Z., 1928. Monit. Zool. Ital., **39,** 137.
CHAIGNE, MARGUERITE, 1934. Bull. Stat. Biol. d'Arcachon, **31,** 1.
CUÉNOT, L., 1907. Arch. Zool. exp. gén., Sér. 4, **7,** 227.
CUVIER, G., 1817. *Mémoire pour servir à l'histoire et à l'anatomie des Mollusques.* Paris (Deterville).
—— 1805. *Leçons d'anat. comp.* Paris.
—— 1837–9. Ibid., 2nd ed. Paris (Duvernoy).
—— 1836–40. Ibid. Bruxelles (apparently reprint of 2nd edition).
DAWES, B., 1930. J. Marine Biol. Assoc., **17,** 75.
DREW, G. A., 1911. J. Morph., **22,** 327.
ENRIQUES, P., 1902. Mitt. Zool. Stat. Neapel, **15,** 281.
FALLOISE, A., 1906. Arch. Int. Physiol., **3,** 282.
GARIAEFF, V. P., 1916. Trav. Soc. Nat. Kharkov., **48,** 1.
GRAHAM, A., 1949. Trans. Roy. Soc. Edinburgh, **61,** 737.
GUTHEIL, F., 1911. Zool. Anz. **37,** 331.
HEINRICH, H., 1904. Z. Naturw., **77,** 1.
HENRI, V., 1903. C. R. Acad. Sci. Paris, **137,** 763.
HIRSCH, G. C., 1925. Z. vergl. Physiol., **2,** 1.

Jordan, H., 1913. *Vergleichende Physiologie wirbelloser Tiere.* Jena (Gustav Fischer).
Joubin, L., 1887. Arch. Zool. expér. gén., Sér. 2, **5** bis, 1.
Kellog, J. L., 1915. J. Morph., **26,** 625.
Marceau, F., 1905. C.R. Acad. Sci. Paris, **141,** 279.
Naef, A., 1923. *Fauna e Flora des Golfes von Neapel,* **35,** *Die Cephalopoden.* Berlin (Friedländer & Sohn).
Owen, R., 1836. Todd's *Cyclopaedia of Anat. & Physiol.,* **1,** 517.
Portmann, Ad., and Bidder, Anna M., 1928. Quart. J. micr. Sci., **72,** 301.
Ranzi, S., 1928. Publ. Staz. Zool. Napoli, **9,** 81.
Romijn, C., 1935. Arch. Néerl. Zool., **1,** 373.
Russell, E. S., and Steven, G. A., 1930. Nature, **125,** 893.
Saguchi, S., 1917. J. Morph., **29,** 1.
Sellier, J., 1907. C.R. Soc. Biol. Paris, **63,** 705.
—— 1910. Bull. Stat. Biol. d'Arcachon, **13,** 1.
Sereni, E., 1928. Boll. Soc. Ital. Biol. Sper., **3,** 707.
Verrill, A. E., 1882. Rep. U.S. Comm. Fish., 1879, 211.
Vigelius, W. J., 1881. Zool. Anz., **4,** 431.
—— 1883. Verh. Kon. Akad. Amsterdam, **22,** 1.
Wigglesworth, V. B., 1931. J. exp. Biol. **8,** 411.
Williams, L. W., 1909. *Anatomy of the Common Squid.* Leiden (E. J. Brill).
Wülker, G., 1910. K. Bayer Akad. Wiss., Math- Phys. Kl., Suppl. Bd. 3, Abh. 1.
Yonge, C. M., 1926. Trans. Roy. Soc. Edinburgh, **54,** 704.

EXPLANATION OF PLATES

Plate I

Fig. 1. Epithelium lining the oesophagus of *Loligo*, fixed Helly, stained Prenant-Hollande. No formative layer is present between the epithelium and the cuticle.

Fig. 2. The same, fixed Zenker. A narrow formative layer is present.

Figs. 3, 4, 5. Epithelium lining the stomach of *Loligo*, fixed Zenker, stained Mallory.

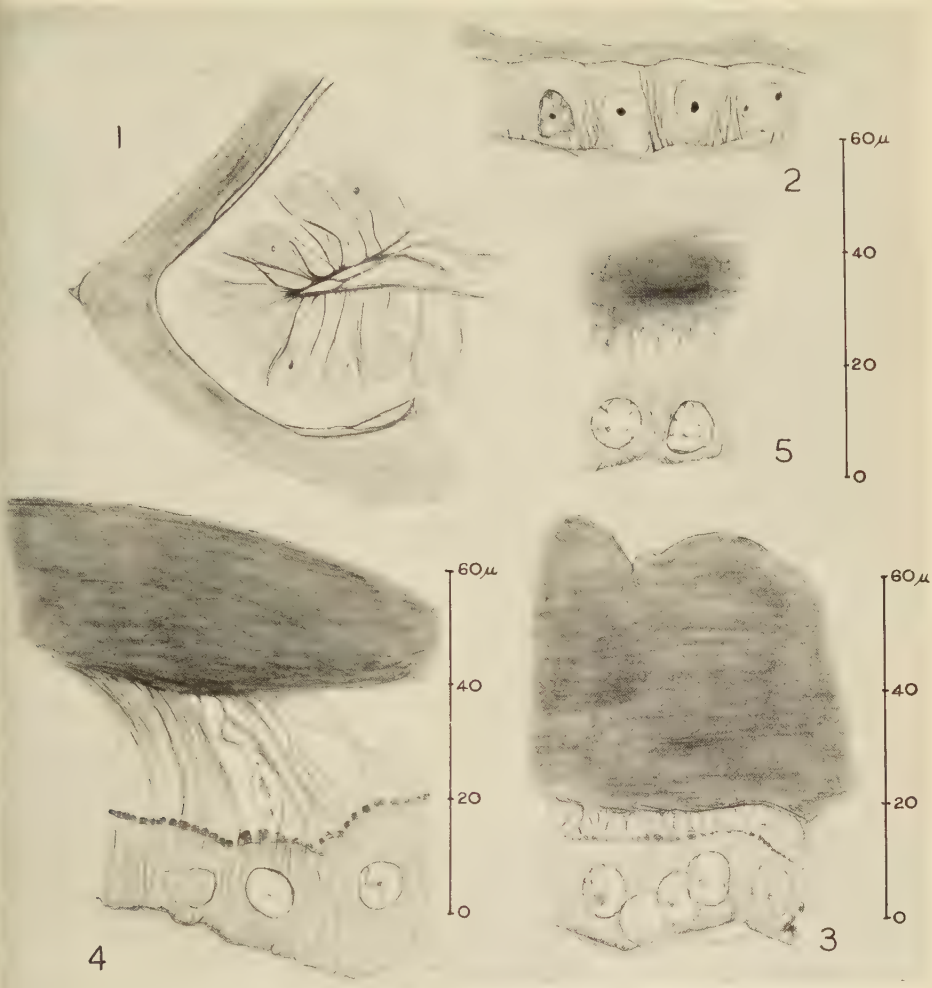
Fig. 3. The fibrillar formative layer is narrow; masses staining red with Mallory present within the right-hand cell.

Fig. 4. The fibrillar formative layer is wide; the red-staining masses are outside the cell-border, and amongst the fibrillae of the formative layer.

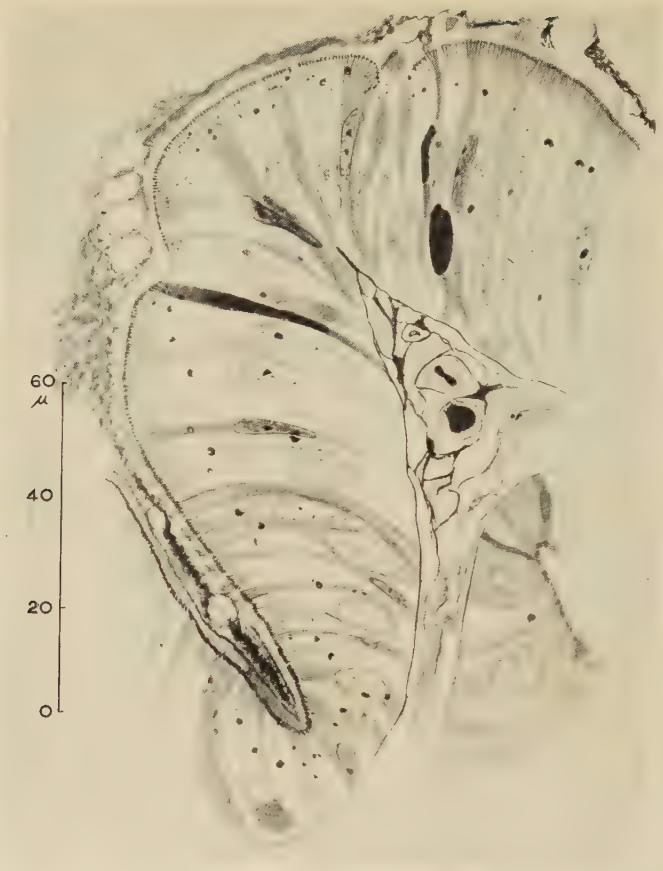
Fig. 5. Active secretion of the formative layer is associated with obliteration of the cell-border.

Plate II

Transverse section through one ridge of a ciliated leaflet of the caecum, fixed Helly, stained iron haematoxylin. The preparation shows the variations in the staining of the cytoplasm, the 'Golgi-like' bodies of the lateral cells and the scattered granules near the surface of the apical cells; and a newly secreted mucus-ball in one of the secondary leaflet grooves.



BIDDER. PLATE I



BIDDER. PLATE II

The Physiology of the Alimentary Canal of *Tyroglyphus farinae*

BY

T. E. HUGHES, M.A.

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THIS investigation into the activities of the gut in *Tyroglyphus farinae Linn.* was undertaken because so little is known of the physiology of the tyroyphid mites. The work was started in 1940 and has been continued as ɔportunity arose since then. It was hoped that the region of secretion of the gestive enzymes, together with the seat of carbohydrate and protein digesɔn and absorption, might be determined. Since the Malpighian tubes in e Tyroglyphidae (Acaridiae) are reduced or absent, it was hoped to find hat region of the gut is responsible for the excretion of nitrogenous waste. n attempt was also made to identify the chief nitrogenous katabolites.

Methods

Mites were cultured on food material stained with various indicators; by is means the pH of the gut was determined subject to protein error, and the ıssage of food was followed. Pig meal and bemax were found to be good ılture media for this purpose. The cultures were kept in cells formed by ıing a glass ring on to a slide with gold size; they were covered by another de held in position by a rubber band. The cells were kept in a dessicator a relative humidity of 90 per cent. Changes in colour of the indicator ɔuld be observed by transparency through the body-wall of the living ıimal.

Cultures were also made on rice starch, potato starch, gluten, and yeast cells localize carbohydrate and protein digestion. Animals cultured on starch ere mounted in a dilute alcoholic solution of iodine. Gluten was used as a ɔtein, because if fed on whole grain the mites attack the germ which is rich this substance. Fibrin and dried red-blood corpuscles were consistently fused by the animals.

Some difficulty was encountered in finding a method of mounting which ɔuld render the mites clear without interfering with the colour of the dicator. The method finally adopted was to drop a live mite into glycerine a slide; on this was placed a coverslip smeared with glycerine jelly. The de was warmed just sufficiently to melt the jelly; this greatly helped the earing, and on cooling the weight of the coverslip was generally found to ve spread the legs without bursting the body. Mounted in this way, the imal could be examined with an oil-immersion lens.

Efforts were made and pursued for several months to dissect out the gut, but without success. As the body-wall of the opisthosoma is punctured, the parenchyma loses fluid and the mite collapses. It appears that mites cultured at a relative humidity of 90 per cent. have a positive internal pressure in this part of the body; this is indicated by their turgid and glistening appearance.

Extracts were made of whole animals collected by standing a beaker of heavily infected material in a half petri dish of water. The mites leaving the culture collected on the water and could be filtered off and washed; after grinding with glass powder, various extracts were made by shaking with the extracting fluid and centrifuging off the solid debris.

Serial sections were cut from paraffin, celloidin, and ester-wax blocks, and stained with Mallory, haematoxylin and eosin, mucicarmine and muci-haematein to investigate the histology of the gut. Some sections were cut after fixation with a 1 per cent. solution of osmium tetroxide to determine the distribution of fats. After fixation with Pasteel and Léonard's fluid (Lisbon, 1936), sections were made and stained with Best's carmine to find the site of glycogen storage. Bauer's reaction was also successfully employed in this respect, as a more specific histochemical test for carbohydrates.

Extracts were made from heavily infected cultures, and specific tests were carried out for various nitrogenous katabolites. Changes in size, opacity, and birefringence of the faecal pellet in its passage through the gut were also investigated. In the following description the term 'dense culture' is employed for cultures containing more than 10^4 mites per gramme in all stages of the life-cycle excluding eggs; young cultures are those containing less than 10^3 mites per gramme, and here the mites are hard to find. In the former, the whole medium appears to be in motion when seen under the binocular microscope, and ultimately such cultures come to consist of exuvia, faecal pellets, and mites only.

Anatomy and Function

The anatomy of the gut of the Tyroglyphids has been described by Michael (1901) and its homologies discussed by Lönnfors (1930); a general description is also given by Vitzthum (1940). The nomenclature used by these and other authors is by no means uniform, and an attempt to correlate it was thought to be useful.

The arachnid gut like that of all Arthropoda is divisible into three regions, a fore-gut lined with chitin, an endodermal mesenteron, and a chitin-lined hind-gut. It is characteristic of the group that the hind-gut—derived from the proctodaeum—is short. The fore-gut becomes differentiated into a buccal cavity and usually a suctorial pharynx of varying complexity, followed by an oesophagus. The mesenteron always develops a system of caeca; and in spiders, pseudoscorpions, pedipalpi, and scorpions, terminates in an enlargement into which the excretory structures discharge, and which connects with the chitinous hind-gut.

In the Acari the fore-gut, if a chitinous lining is to be taken as any guide, certainly gives rise to the buccal cavity and pharynx. The oesophagus is only reported as having a lining of chitin in the prostigmatic Trombidiformes (Sig Thor, 1904), the Halacaridae (Thomae, 1925), and the Analgesidae (Lönnfors, 1930). Other authors have reported the oesophagus, in the forms studied by them, as having no chitinous lining and consisting of a thin epithelium. Thus the homology of the oesophagus of the Acari with that of other arachnids, where it is demonstrably part of the fore-gut, is by no means clear. The mesenteron has been ascribed various limits, though the term, when used, has been most frequently restricted to that part which bears the caecal outgrowths. The term hind-gut, or Enddarm of German authors, has also been used in a loose sense to cover the regions of the gut which are not lined with chitin, and which properly should be regarded as part of the mesenteron.

It is proposed here to limit the fore-gut to the buccal cavity, pharnyx, and oesophagus, and to regard the alimentary canal between the oesophagus and the chitinous region of the rectum as being mesenteron. The term rectum is restricted to that part of the posterior end of the alimentary canal which is lined with chitin; the mesenteron then becomes divisible into an anterior stomach provided with caeca, an intermediate colon, and finally a post-colon.

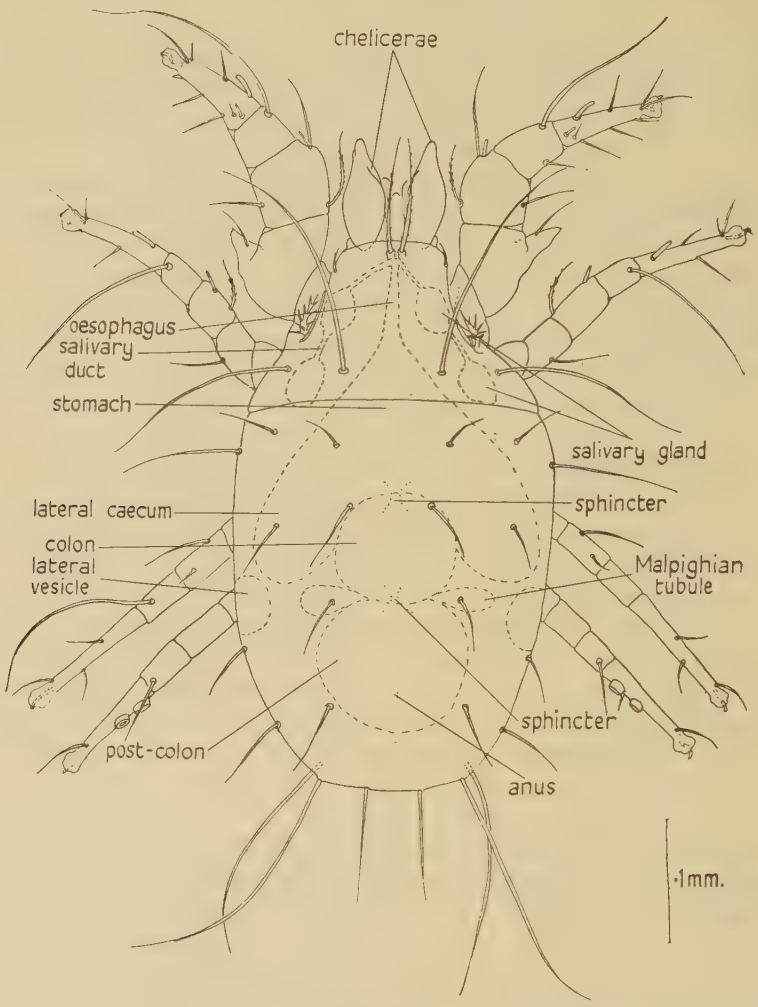
The gut of *T. farinae* is of the typical tyroglyphid type (Text-fig. 1). The mouth lies below the insertion of the chelicerae; and the buccal cavity, which is bounded above by the very short epistome and the bases of the chelicerae, and below by the fused bases of the pedipalps, leads into a pharynx.

The pharyngeal wall is heavily chitinized and crescentic in cross-section with the concavity dorsal. The dorsal wall of the pharynx can be raised by means of five pairs of dilator muscles which have their origins on the internal skeleton of the gnathosoma, which here projects back from the sub-cheliceral shelf into the gnathosoma as a pair of rods. The muscles are inserted on to the roof of the pharynx near its lateral edges. Starting behind the second dilators, four constrictor muscles alternate with them, running transversely from horn to horn of the pharyngeal crescent. Food appears to be seized by the chelicerae and pushed into the buccal cavity through the mouth, the free palpal parts of the pedipalps probably assisting in this. The action of the pharyngeal muscles is to raise the roof of the pharynx by means of the dilator muscles and to allow food to be pushed back into its lumen. By successive contraction of the constrictors from before backwards, the contents of the pharynx, provided they are not too dense or solid, could be squeezed backwards into and along the narrow oesophagus. Whilst the process is going on, the pharynx is held firm by a mid-ventral attachment to the body-wall, and lateral connective tissue attachments from its edges to the ventrolateral body-wall.

Michael (1901) described paired salivary glands in the Tyroglyphidae as opening by a short common duct into the posterior end of the buccal cavity through its roof. He said that the ducts were very difficult to follow. All authors, with the exception of Lönnfors, are agreed that the Acari in general

possess such salivary glands (Vitzthum, 1940). In *T. farinae* the ducts of th glands open into the posterior angles of the buccal cavity (Text-fig. 2).

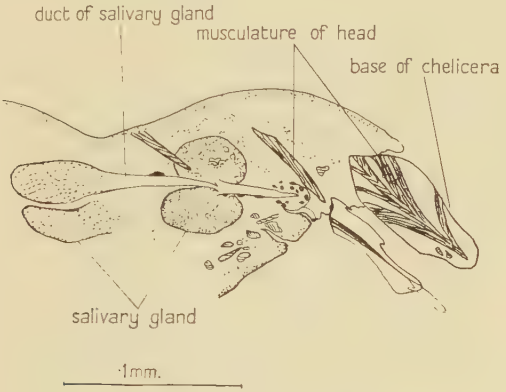
In *T. farinae* the food, whether starch grains from flour or pieces broke off whole grain or coarse meal by the chelicerae, is always relatively dry, an



Text-fig. 1. Dorsal view of *Tyroglyphus farinae* to show the alimentary canal.

it has to traverse a long pharynx and longer oesophagus. This penetrates the solid central nervous system, and has walls which are thin and certainly devoid of muscles. It is improbable that such a passage could be achieved without the secretion of some lubricant, leaving aside the question of a digestive function for the saliva. It is difficult to understand, too, how the pharyngeal apparatus could deal with anything other than a fluid or semi-fluid bolus.

The salivary glands consist of two paired masses of cells on each side of the body, each consisting of a few cells only. The cytoplasm of these cells after fixation with Léonard and Pasteel's fluid (Lison, 1936) contains small granules which stain with eosin; after fixation in alcoholic Bouin the cytoplasm (Text-fig. 2) contains many small vacuoles giving it a spongy appearance. In addition, each cell contains a large vacuole into which projects the nucleus thinly covered with cytoplasm. Each discharges into a common duct the thin walls of which are composed of flattened cells. Mucihaematein does not stain the general cytoplasm but does colour the small granules; mucicarmine gives a pale staining of the entire gland.

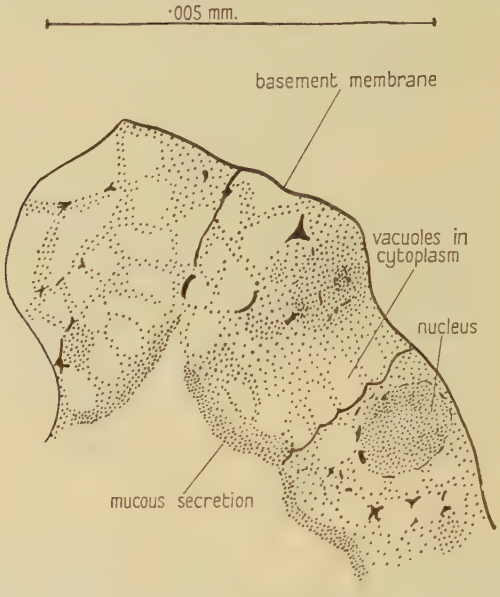


Text-fig. 2. L.S. head.

The pharynx, which supplies the mechanical force to propel the food into the stomach, passes almost at right angles into the thin-walled oesophagus. As in *Glycyphagus domesticus* (Hughes and Hughes, 1938) the oesophagus projects a short distance into the lumen of the stomach, with which it joins antero-ventrally. The wall of the oesophagus consists of a thin epithelium with a very tenuous lining of chitin. The inner lining often shrinks away from the wall in section where the oesophagus is empty. It would appear that in *T. farinae* the oesophagus is part of the fore-gut.

The stomach, into which the oesophagus opens, bears post-laterally a pair of blunt caeca. These extend back below and slightly to each side of the succeeding part of the mesenteron or colon, from which the stomach is separated by a sphincter. The stomach itself is pear-shaped with the narrow end forwards. Its walls consist of a single layer of cells bounded externally by a heavy basement membrane. These cells, though all similar, are not identical (Text-fig. 3); they have granular cytoplasm, and contain many vacuoles, the nuclei being large and lying near the basement membrane. None of them is a goblet cell, nor are the vacuoles conspicuously near the lumen of the gut. The cells of the anterodorsal stomach wall and of the ends of the blunt caeca, though of the same general form, are taller and tend to project into the lumen; such cells would correspond to the pre- and post-ventricular

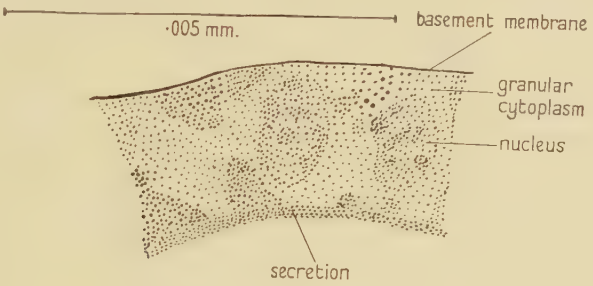
glands of Lönnfors (1930). In sections fixed in Léonard and Pasteel's fluid picro-chlor-formalin, or Flemming's solution and stained with Mallory's triple stain, a layer of deep-blue substance is seen along the free borders of the cells, but not in the vacuoles. In sections stained with Masson's acid fuchsin and light green, the contents of the stomach are green; its wall cells show no discrete spherules, only a diffuse staining. The same result is given with mucicarmine and mucihaematein. This would suggest the secretion of



Text-fig. 3. Wall of stomach and caeca.

mucus, probably containing a digestive enzyme, by passage through the cell surface. The taller cells at the tip or anterodorsal region of the caeca, often contain red-staining spherules, which seem similar to those recorded in the intestinal wall of *Peripatopsis* by Manton and Heatley (1937) and regarded by them as protein reserves. If animals are fed on food stained with indicators or vital stains, these cells in particular become filled with small vacuoles coloured with the dye. Lönnfors immersed Analgesidae in aqueous solutions of vital stains without any very marked coloration, but he does record that neutral red was taken up by the caeca. *T. farinae* was fed on coarse meal or on gluten stained with universal indicator, litmus, phenol red, or neutral red. 'Universal' indicator was always orange-yellow in the stomach, although it stained the meal red and the gluten green. Litmus was always red in the stomach and caeca; phenol red was yellow and neutral red remained red. From these results it would appear that the pH of the stomach and its caeca lies between 5·0 and 6·0. Animals fed in this way showed a vacuolization of the cells of the stomach wall, particularly at the tips of the caeca and anterodorsally; this gives the cells concerned the appearance of bunches of grapes.

When viewed under an immersion lens it was possible to see small granules in vibratile motion in each vacuole. It was not found possible to determine the nature of these granules. There seems little possibility that these vacuoles are not absorption vacuoles, since they occurred whatever dye was used. Although basic dyes, such as neutral red, may cause vacuolization, acidic dyes like phenol red do not; moreover the fact that a variety of food dyes found in proprietary pudding- and custard-powders also appear in the cells in a similar way, is itself an indication that these cells are actively engaged in absorption. Such vacuolated cells are not confined to the tips of the caeca and antero-dorsal stomach wall, but are most frequent there.



Text-fig. 4. Wall of colon.

There are no intrinsic muscles in the walls of the stomach and its caeca, but high in its posterior wall and between the caeca, is a sphincter which leads into the colon. This is spherical in shape and communicates post-ventrally by another aperture, guarded by a sphincter, with the post-colon. The wall of the colon is thinner than that of the stomach and not vacuolated (Text-fig. 4). Its contents are often solid as contrasted with the fluid contents of the stomach. In animals fed on dyed food, a coloured bolus may be visible and the walls are always diffusely stained in contrast to the very obvious vacuolization of the stomach walls. During starvation of such animals, dye can be seen in the colon walls and very diffusely around it some time after it has entirely disappeared from the stomach and its caeca. 'Universal' indicator is yellow in the colon, litmus changes from red to purple, phenol red is yellow, and neutral red remains red. The pH therefore rises until it is above 7·0 but below 8·0. The contents of the colon may be in either of two conditions: fluid like that of the stomach, or apparently fairly solid and surrounded by a thin peritrophic membrane. This, since it appears in the colon, is presumably secreted by its walls. This peritrophic membrane is colourless in life but stains blue with haematoxylin. Coloration of the colonic wall, the formation of a bolus of greater opacity than the stomach contents that enter it, and which retains its form in passing the sphincter into the post-colon, indicate that the functions of the colon are the absorption of the more fluid part of the material passing into it, as well as the production of the peritrophic membrane. This protects its walls and those of the post-colon from the solid bolus which is thus produced. In addition, the walls of the colon may secrete some other

substance than the peritrophic membrane, since the pH of the contents rises whilst in the colon. The peritrophic membrane is mentioned by Vitzthum (1940), but Lönnfors and earlier authors did not apparently see it in the species they studied.

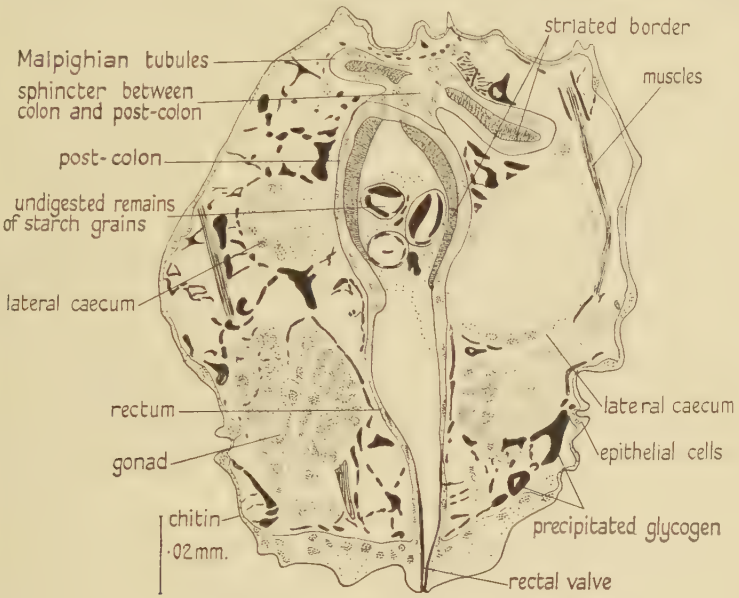
In animals taken from a dense culture on wheat germ, where there is extensive fouling of the food with faecal pellets, the contents of the colon are practically always visible as a pellet. This is much denser than that found in individuals from young cultures where the food is clean; moreover, with polarized light, the pellet is birefringent and has a yellow colour, whereas if the food is clean, the contents are rarely birefringent except for easily recognizable starch grains. In animals from cultures on dried yeast or gluten, the colonic pellet closely resembled that of animals from young wheat-germ cultures, the pellet being rarely birefringent. On the other hand, in such animals and also in animals from dense cultures, the parenchyma of the opisthosoma is usually crowded with birefringent crystals in Brownian movement within their vacuoles. It would seem that in animals, the food of which is contaminated with faecal material, the concentration of this can become such that the absorption of water in the colon is sufficient to precipitate some katabolite present in crystalline form. In animals kept on food relatively free from such contamination, however, the concentration of such material in the colon is very rarely high enough to cause precipitation, although crystals accumulate in the parenchyma if the substrate is richer than usual in protein. The crystals, then, are presumably connected with the protein metabolism of the animal and may well be of an excretory nature. The greater density of the colonic contents in the case of dense culture animals is due not only to the precipitation of crystals, but also to the fact that the animals in such cultures are reduced to eating the more cellular constituents. As any adhering starchy endosperm or embryo has already been consumed there is a greater proportion of unutilizable material from the food itself taken in.

No intrinsic muscles were seen in the region of the gut, nor have they been recorded by Michael (1901) in Tyroglyphidae or by Lönnfors (1930) in Analgesidae, although Vitzthum (1940) speaks in a general way of the gut of acarids as having a muscle-coat posterior to the stomach. Since Michael, using the same methods, described an extensive musculature on the colon and on the stomach of Oribatidae, it seems reasonable to suppose that there is considerable variation among the Sarcoptiformes in this respect.

From the colon, the gut contents in the form of a bolus surrounded by a peritrophic membrane, pass into the post-colon. This passage can sometimes be observed in animals mounted alive in dilute glycerine. It is brought about suddenly by contraction of dorso-ventral muscles of the body coinciding with relaxation of the sphincter between colon and post-colon. This may coincide also with the extrusion of a faecal pellet, the anal valves being opened by their own muscles. However, the post-colon is frequently found empty.

The post-colon, like the colon, is spherical, but ventrally passes by a narrow opening not guarded by a sphincter (Text-fig. 5) into the chitin-lined

rectum. The walls of the post-colon are formed of an epithelium with a deep striated border apparently composed of long narrow vesicles (Text-fig. 6). The boundaries of the cell bodies are very indistinct and their cytoplasm contains small vacuoles. These appear coloured in animals fed on dyed foods and then starved until the gut is empty, the presence of a faecal pellet making it impossible to see the walls very clearly. In live mounts in glycerine, the striated border shows as a clear homogeneous zone round the faecal pellet, the cell-bodies as a denser surrounding ring. Nalepa (1884) considered this

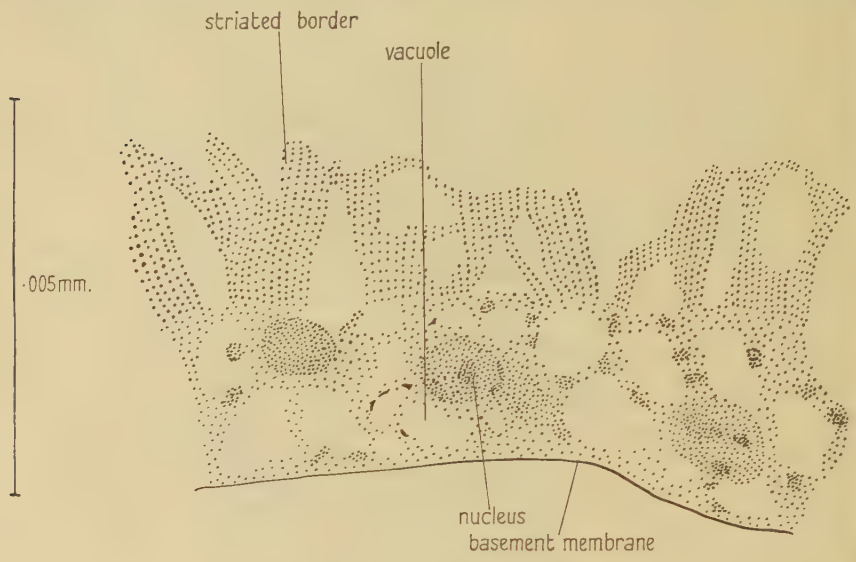


Text-fig. 5. T.S. in the region of the rectum.

region of the gut to be excretory and Michael (1901) was of the same opinion. No excretory granules have been found in its walls or outside the peritrophic membrane which surrounds the bolus; nor have I ever seen any reference to such granules or crystals in the literature dealing with the Tyroglyphidae. Its real function is indicated by the presence of the coloured vacuoles mentioned previously, which would suggest a final absorption of water rather than the excretion of a coloured fluid, since the faecal pellet is a dry, solid structure. The histological structure of the post-colon, which resembles that of the lower ends of the Malpighian tubules of an insect such as *Rhodnius* (Wigglesworth, 1931), also suggests that its true function is a final absorption of water. This must be an important function of the gut in an animal like *T. farinae* which lives on food of relatively low water content without access to fluid water.

Further evidence is obtained by considering the size of the colonic pellet when it has been surrounded by a peritrophic membrane and therefore has a sharp outline. This enables its diameter to be measured and compared with

the diameter of an unvoided faecal pellet in the same animal. Two facts are at once noticeable. In the case of animals taken off clean food, the faecal pellet in the vast majority of cases is smaller than that in the colon, and its density and opacity to transmitted light are greater. In animals from dense cultures, however, both faecal and colonic pellets are opaque. Moreover, when the presence of birefringent crystals is investigated, it is found that in animals from cultures on dried yeast or gluten, the faecal pellet is usually



Text-fig. 6. Wall of post-colon.

entirely or partially birefringent, and on wheat germ it, too, may show birefringent patches. In animals from dense cultures, however, both faecal and colonic pellets are always strongly birefringent. These facts indicate that the post-colonic pellet undergoes further drying, which is sufficient to bring out of solution any of the crystalline substance which originally may have been present in solution. The fact that the crystalline substance appears within the peritrophic membrane surrounding the pellet, suggests that it was already in this position when the bolus entered the post-colon, so that presumably excretion of this substance has already taken place in the colon or possibly even in the stomach. No change in pH was detected in this region of the gut, so that the actual method of precipitation of the crystals is different from the precipitation of uric acid in *Rhodnius* (Wigglesworth, 1931).

The post-colon opens freely into the rectum which in transverse section appears as a bilaterally compressed vertical slit. It is lined with chitin, which is thickened distally to form the rectal valves and then passes over the edge of the anal opening into continuity with the exoskeleton. The cellular layer which secretes this chitin appears as a syncytium continuous with the epithelium of the body-wall. The muscles operating the anal valves are inserted

just inside the anal opening and run from the valves to the ventrolateral body-wall, close to the ventral side of the body. The faecal pellet never appears to be retained in this region of the body, the sole function of which is to afford it an exit.

Malpighian tubules were first described in *T. farinae* by Berlese (1897). They are a pair of short, blunt tubules standing out at right angles to the constriction between colon and post-colon. It appears that they can discharge any contents they might accumulate only when this sphincter relaxes. Warren (1944) described the paired Malpighian tubules of a species of *Urodinychus*, and was of the opinion that there was no communication between them and the gut. Granules which he saw in them and called guanine bodies passed through their cytoplasm and that of the gut-wall to reach its lumen. In *T. farinae* the tubules consist of a few cells only which histologically resemble those of the post-colon. No granules have been observed in any part of them.

Various tests were made on the animal, its faecal pellets, and on extracts from cultures in order to ascertain the nature of the crystalline excretory products. Water extracts from 50 gm. of dense cultures extracted in 500 c.c. of fluid, and from faecal pellets from the 6-cm. diameter cellophane covers of culture jars to which some thousands of faecal pellets adhered, extracted in 10 c.c. of fluid, both gave negative results with the urease tests for urea. Weil's test and the picric acid test for creatinine were also negative. When similar extracts made with 2 per cent. sodium carbonate were saturated with ammonium chloride and the resultant precipitate subjected to the murexide test, an orange colour was obtained. Faecal pellets were collected from the cellophane covers and the murexide test applied to them in the cavity of a hollow-ground slide, and again an orange colour resulted. It would appear from this that uric acid is not produced as a nitrogenous katabolite. The orange colour might be due to guanine or xanthine (Cole, 1933).

Animals were taken from cultures on gluten and dense cultures on wheat germ, all having the parenchyma of the opisthosoma crowded with crystalline bodies. These animals were incubated overnight at 56° C. in various solutions and then examined microscopically in a drop of the solution next day. The crystals were found to be soluble in dilute sulphuric acid and in caustic potash, but insoluble in organic solvents or a solution of piperazine. Insolubility in piperazine is given by Lison (1936) as a distinguishing test between uric acid and guanine.

Faecal pellets treated in the same way lose their birefringence in sulphuric acid and caustic potash, but not in cold dilute hydrochloric acid or piperazine. If a heavy culture is sifted a little at a time through fine silk material, a grey dust containing a high proportion of faecal pellets can be obtained. If such material, or dense cultures themselves, are extracted over a water bath with 5 per cent. sulphuric acid, guanine can be precipitated by saturation with ammonia. After repeated solution and precipitation by ammonia to free it from other substances, the final precipitate was dissolved in a small quantity

of hot hydrochloric acid and filtered hot. On cooling, small crystals separated out which redissolved on heating. A white precipitate could be obtained from the solution in hot hydrochloric acid by the addition of 10 per cent. solution of metaphosphoric acid. This precipitation by metaphosphoric acid was first used by Wulff (1893) for quantitative extraction of guanine.

Since tests carried out on the granules in the animals, on faecal pellets and on extracts from faecal pellets or dense cultures all give positive results for such tests for guanin as can be applied, there seems nothing inherently improbable in the view that this is the chief nitrogenous katabolite and that uric acid is not produced in a quantity detectable by the means employed.

Experiments were also carried out to determine the optimum pH for the digestion of starches and protein. Tubes were set up in duplicate each containing 3 c.c. of a buffer solution in steps of 0·2 pH and 1 c.c. of a filtered starch solution in saturated sodium chloride. To one series of tubes was added 1 c.c. of water to each tube; to the other series 1 c.c. of a water extract (made as described on p. 46) of whole animals was added. After the addition of a drop of toluene, the tubes were plugged with cotton-wool and incubated at 25° C. for 48 hours. The sugar in each tube was estimated by the Jensen-Hagedorn method, and the difference between any pair of tubes at the same pH taken as a measure of the digestion which had occurred. These experiments gave a maximum of sugar of pH 5·4.

Proteolytic enzymes present in such extracts were tested in similar experiments, using 2 c.c. of a 1 per cent. gelatine solution in place of the starch solution. After 48 hours' incubation the amino-acids present were estimated by Sörensen's method. The optimum pH was found to be 5·6.

Proteolytic enzymes may be precipitated by safranin (Robertson, 1907) and neutral red (Marston, 1923); the activity of such precipitates was demonstrated by Marston and Holzberg (1913). When such precipitates were prepared from extracts of whole animals and used in similar experiments, they had an optimum pH of $5{\cdot}6 \pm 0{\cdot}2$.

A weak lipase can also be shown to be present in water extracts, by using an emulsion of olive oil coloured with alkaline phenolphthalein. The salivary secretion is difficult to investigate, and careful observation of mites on food dyed with indicators does not suggest that there is any pouring out of the saliva on to the food.

Storage of food reserves was investigated by using Bauer's method and Best's carmine for glycogen and by osmic fixation for fat. The parenchyma, in which the internal organs lie embedded, contains large quantities of glycogen (Text-fig. 5), as can be shown by either method. The fluid, which escapes if the opisthosoma is punctured, also contains glycogen. Fat does not appear to be metabolized to any extent by these animals, since there was no indication of fat droplets in the parenchyma comparable with the amount of glycogen, deposits of which are very heavy. In sections stained by Bauer's method it is possible to see starch grains in stomach, colon, and post-colon in

various stages of digestion. This, together with the detection of starch grains by birefringence in the colon and post-colon, suggests that much carbohydrate material escapes digestion.

Discussion

Lönnfors (1930) called the salivary glands in the Analgesidae and *Carpoglyphus passularum* (*lactis*) studied by him pseudosalivary glands, and considered that they opened above the coxa of leg 1. Grandjean (1937), in his description of the podocephalic canal of various mites based on a study of whole mites mounted in lactic acid solution, speaks of the chitinous ducts of two glands opening in the region of the pseudostigmatic organ. Grandjean states that they are very difficult to see and that he has assumed without proof that they are homologous with certain ducts present in *Otodectes cynotes*: he also says in a footnote:—

'Il vaut mieux admettre pour le moment, qu'un doubte subsiste parceque le "fil" ne semble pas toujours creux, son apparence étant quelquefois celle d'un tendon. Du moins en est il aussi dans les préparations traités à chaud par l'acide lactique. Avant ce traitement, ou un traitement analogue, le fil est à peine discernable. Je n'ai jamais constaté en observant des acariens vivants, qu'un tube ou organe plein d'air aboutisse á la fossette supracoxale.'

The position of the suspected opening above leg 1, in the supracoxal groove, is easily checked in section in *T. farinae* because of the characteristically shaped pseudostigmatic organ which is strongly birefringent in polarized light. I have not been able to trace any duct from the salivary glands to this region either in transverse, longitudinal, or horizontal sections. Grandjean (1937, Text-fig. 1B) figured the possible openings as a straight oblique line above and slightly anterior to the pseudostigmatic organ, and a smaller crescentic line just above its base. I have examined *T. farinae* in 60 per cent. lactic acid solution in lateral view; the straight line is clearly visible, but I have been unsuccessful in seeing the smaller crescentic one. In horizontal sections a muscle is seen running ventral to the salivary glands from the region of convergence of the apodemes of the legs to the lateral body-wall in front of and above the pseudostigmatic organ. It would appear probable that the straight line on the exoskeleton which Grandjean saw is the tendon attachment of the muscle.

From personal observation both *Bdellonyssus bacoti* among the Parasitiformes and *Cheyletus eruditus* among the Trombidiformes have glands which open anteriorly, although in both these cases the glands assume a greater complexity and larger proportion than in *T. farinae*. This is presumably correlated with their feeding habits. *B. bacoti*—which feeds on mammalian blood—must, like other blood-sucking Arthropoda, be able to produce some anticoagulant and laking secretion in fairly large quantities, since the volume of a meal is large relative to the mite. *C. eruditus* feeds on the soft parts of other mites and insects, which are sucked dry leaving the empty exoskeleton.

It presumably injects into the prey some salivary secretion which carries out a general maceration if not digestion of the viscera and muscles, in order to allow the passage of food along the very narrow oesophagus. It would seem unlikely that the tyroglyphids feeding on relatively dry substances would have lost glands which are present in the two other groups. The possibility of a regurgitation of fluid along the oesophagus from the stomach, in order to facilitate the passage of food, does not seem very likely. The oesophageal projection into the stomach is of common occurrence. It appears to form a valvular apparatus for the prevention of such regurgitation. Lönnfors, however, did not find any similar structure in the Analgesidae.

Reuter (1909) divided the alimentary canal of the Acari into four types on the basis of variation in structure of the stomach. The Sarcoptiformes belong to his type 1 which, he stated, has a round or pyriform stomach provided with one pair of lateral caeca. The primitive condition is taken by Lönnfors (1930) as having three pairs of caeca (type 3 of Reuter), anterior, median, and posterior, such as is now found in the Parasitidae. Lönnfors considers that the sarcoptiform type has been derived by loss of all but the posterior pair of caeca. The anterior pair has been reduced to the proventicular glands described by Michael (1883) in the Oribatidae, which open into the stomach in a position corresponding to the anterior caeca where present. In the Analgesidae which he studied, still further reductions were traced by Lönnfors (1930). He arranged a series of species where first the proventricular glands occur as a pair of patches of cells on the anterior wall of the stomach, and finally as a single median patch above the opening of the oesophagus, this last state being arrived at by the fusion of the originally paired rudiments. The presence of large cells anterodorsally in the stomach wall of *T. farinae* supports this view. Similar large cells standing out above the general epithelium have been recorded in *Ixodes* sp. (Nordenskiold, 1905), *Halarachne* (Steding, 1923), *Pediculopsis graminum* (Reuter, 1909), and in Tyroglyphidae (Michael, 1901, and Nalepa, 1884). In *G. domesticus* the stomach and its caeca were found to contain cells whose free ends after forming vesicles become nipped off into the lumen of the gut (Hughes and Hughes, 1938). Nalepa has suggested that such cells form the basis of the excretory system. No such cells have been found in *T. farinae*, and the large cells of the stomach and caeca show every sign of being concerned with the absorption of the products of digestion rather than with the liberation of any product either digestive or excretory.

The colon, post-colon, and Malpighian tubules appear to form a functional unit in the system, in that in the colon and post-colon the solidification of the faeces takes place and guanine excretory products become recognizable in the gut contents. In this process the Malpighian tubules appear to play singularly little part. Vitzthum (1940) says that the Malpighian tubules of such tyroglyphids as possess them are always devoid of contents. The appearance in living animals certainly suggests this in sharp contrast to such forms as the gamasids where the Malpighian tubules are visible as white lines

through the dorsal body-wall. Similarly the single median excretory tubule in such trombids as *C. eruditus* makes a definite white line along the middle of the opisthosoma. In sections of *B. bacoti* and of *C. eruditus*, these excretory organs are seen to be full of a crystalline substance –strongly birefringent like that occurring in the faecal pellet of *T. farinae*. In *T. farinae* such crystals never occur in the sections of the Malpighian tubules even when they are densely packed in the parenchyma. Moreover, in many related forms such as *G. domesticus* the Malpighian tubules are known to be absent. It would appear that in *T. farinae* they are functionless as organs of excretion and that the excretory activity has been taken over by some part of the gut-wall. It is at least suggestive that in the Trombidiformes the gut ends blindly, and the single median excretory tubule corresponds in position and point of opening to the exterior with the posterior part of the alimentary canal. The food-containing part of the gut in these forms, from its caecal arrangements and what is known of its histology, corresponds to the stomach. It seems at least possible that the colon, post-colon, and rectum have become separated off to form the median excretory apparatus. Vitzthum (1940) states that the nitrogenous excretion of all mites consists of guanine, but that in the Tyroglyphidae urates may be found as well. Nalepa (1884) considered that non-crystalline urates may be found in the faecal pellets of *T. longior*. Leydig (1859) reported finding uric acid in a mite. Guanine has been demonstrated in spiders by Vafropala (1935), in scorpions by Davy (1857) and Marchall (1890). Warren (1944) spoke of guanine bodies in the Malpighian tubules of *Urodinychus* sp. but does not record having applied any specific tests to them. In *T. farinae* guanine seems to be the only considerable excretory product of nitrogen metabolism, and this is in agreement with the widespread occurrence of the substance within the Acari and within the Arachnida as a class.

Acknowledgements

My thanks are due to Professor H. G. Jackson and to Professor Alastair Graham for much helpful advice; and to the Waller Research Fund for lending me a binocular microscope, without which the manipulation of these mites, during the earlier part of this work, would have been impossible.

Summary

1. The pH of the stomach and its caeca lies between 5·0 and 6·0, that of the colon changes to the alkaline side of 7·0 but remains below 8·0. The pH of the post-colon is between 7·0 and 8·0. All these measurements are subject to protein error.

2. The stomach and its caeca are regions of:

(*a*) absorption;

(*b*) secretion of a mucus containing a digestive enzyme;

(*c*) digestion of carbohydrate and protein.

3. The optimum pH of carbohydrate digestion is 5·4 and of protein digestion 5·6±2.

4. The thin-walled colon regulates the pH of the food bolus and is the site of formation of a peritrophic membrane and some absorption.

5. The post-colon is the site of further absorption of water and solidification of the faecal pellet.

6. Excretory substances are first recognizable in the colon; they do not appear to be produced by the Malpighian tubules or post-colon.

7. The chief nitrogen katabolite is guanine.

8. There is an accumulation of nitrogenous katabolites in the parenchyma of the body in animals on a high-protein diet.

9. The powers of excretion of nitrogenous waste appear to be poor.

10. The main food reserves are of glycogen in the parenchyma; fat is not stored to any extent.

11. The salivary glands produce a mucus which serves as a lubricant for the food in its passage through the pharynx and oesophagus.

LIST OF REFERENCES

Berlese, A., 1897. 'Ricerche sugli organi e sulla funzione della digestione negli acari.' Rivist. Pat. Veg., **5**, 129.

Cole, S. W., 1933. *Practical Physiological Chemistry*. Cambridge (Heffer).

Davy, J., 1857. 'On the urinary excretion of fishes with some remarks on this secretion in others classes of animals.' Trans. Roy. Soc. Edin., **21**, 543.

Grandjean, F., 1937. 'Sur quelques caractères des Acaridiae libres.' Bull. Soc. Zool. Fr., **62**, 388.

Holzberg, H. L., 1913. 'A new method of isolating trypsin.' Jour. biol. Chem., **14**, 335.

Hughes, T. E., and A. M., 1938. 'The internal anatomy and post-embryonic development of *Glyphcyphagus domesticus* de Geer.' Proc. Zool. Soc. B, **108**, 715.

Leydig, F., 1859. 'Ueber Haarsack Milben und Kratzmilben.' Arch. Naturgesch., **25**, 338.

Lison, L., 1936. *Histochimie animale*. Paris (Gauthier Villars).

Lönnfors, F., 1930. 'Beiträge zur Morphologie der Analginen.' Acta Zool. Fenn., **8**, 1.

Manton, S., and Heatley, N., 1937. 'Studies on the Onychophora II.' Phil. Trans. Roy. Soc. B, **227**, 411.

Marchal, P., 1890. 'L'acide urique et la fonction rénale chez les invertébrés.' Mém. Soc. Zool. Fr., **3**, 31.

Marston, H. R., 1923. 'The azine and azonium compounds of the proteolytic enzymes.' Biochem. Jour., **17**, 851.

Michael, A. D., 1883. *British Oribatidae*. London (Ray Soc.).

—— 1901. *British Tyroglyphidae*. London (Ray Soc.).

Nalepa, A., 1884. 'Die Anatomie der Tyroglyphen.' Sitz. Akad. Wiss. Wien, Abt. 1.

Nordenskiöld, E., 1905. 'Zur Anatomie und Histologie von *Ixodes reduvius*.' Zool. Anz., **28**, 478.

Reuter, E., 1909. 'Zur Morphologie und Ontogenie der Acariden mit besonderer Berücksichtigung von *Pediculopsis graminum*.' Acta Soc. Sci. Fenn., **36**, 4, 1.

Robertson, T., 1907. 'Studies on the chemistry of the ion proteid compounds.' J. biol. Chem., **2**, 317.

Steding, E., 1923. 'Zur Anatomie und Histologie der *Halarachne otariae*.' Z. wiss. Zool., **121**, 442.

Thomae, H., 1925. 'Beiträge zur Anatomie der Halacariden.' Zool. Jhb. Anat., **47**, 155.

Thor, S., 1904. 'Recherches sur l'anatomie des acariens prostigmatiques.' Ann. Sci. nat. (zool.), **19,** 1.
Vajropala, K., 1935. 'Guanine, the excreta of arachnids.' Nature, **136,** 145.
Vitzthum, H. Graf., 1940. 'Bronns Klassen und Ordnungen des Tierreiches, Acarina.' Leipzig (Becker & Erler).
Warren, E., 1944. 'Observations on the anatomy and histology of a myrmecophilous mite and an account of its sporozoan parasites.' Ann. Natal Mus., **10,** 359.
Wigglesworth, V. B., 1931. 'Excretion in *Rhodnius prolixus*.' J. exp. Biol., **8,** 411.
Wulff, C., 1893. 'Beiträge zur Kenntniss der Nucleinbasen.' Zeit. physiol. Chem., **17,** 468.

The Outer Layers of the Cuticle in the Cockroach *Periplaneta americana* and the Function of the Oenocytes

BY

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With one Plate

IT is now recognized that the 'epicuticle' of insects is a complex structure made up of several layers (Wigglesworth, 1948*b*). A primary 'cuticulin' layer of tanned lipoprotein is covered by a layer rich in silver-reducing substances (the 'polyphenol layer') on which is deposited a 'wax layer' protected, finally, by a layer of 'cement' poured out by the dermal glands at the time of moulting.

To what extent the wax permeates the other layers of the 'epicuticle' has not been clearly determined. This may well vary from one insect to another. As was shown by Dusham (1918), the cockroach produces a soft wax which is freely exposed on the surface of the cuticle; it is presumably secreted throughout the life of the insect. The function of this wax, like that of the covered waxes of other insects, has been proved to be the protection of the insect from water loss (Ramsay, 1935; Beament, 1945; Wigglesworth, 1945).

The object of the present work was to study the origin of the cuticular wax in the cockroach and its relation to the cement layer, if indeed a cement layer does exist in this insect.

Dermal Glands and the Cement Layer

The dermal glands in the cockroach *Blatta orientalis* have been described by Konček (1924) and Stanislavskij (1926) as consisting of: (i) simple glands present only in the male, abundant on the tergites of the abdomen, occurring sparsely on the sternites; and (ii) alveolar glands, in which large numbers of unicellular glands of the same type as (i) discharge into the sac-like invaginations between the fifth and sixth abdominal tergites of both sexes and into a fold of the sternites beneath the fifth abdominal ganglion.

If the cuticle of the adult *Periplaneta americana* is mounted in glycerol or gum syrup without staining, and examined with the microscope in surface view, it is possible to detect the openings of dermal glands in the tergites and sternites of both sexes, although they are far more numerous in the tergites of the male (Text-fig. 1, A, B).

If the intact insect is immersed in boiling chloroform and then in ammoniacal silver hydroxide (Wigglesworth, 1948*a*), the linings of these dermal

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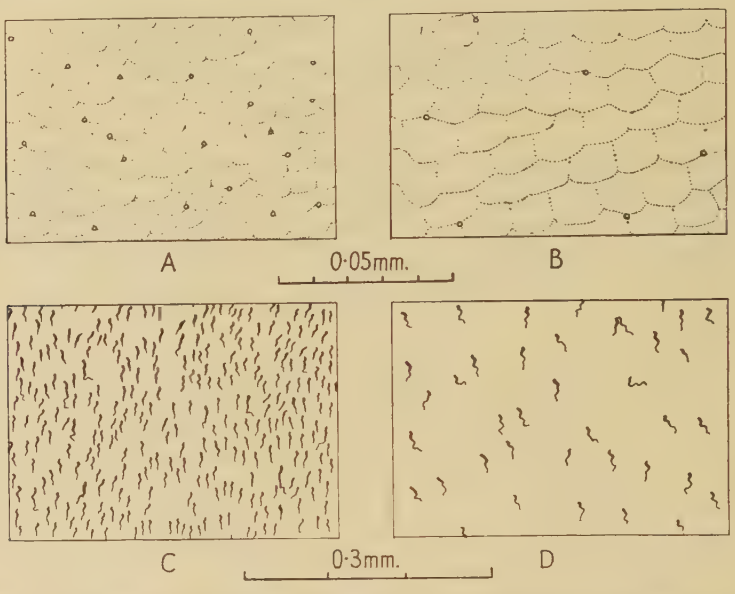
glands are darkened; and the extraordinary abundance of the glands, particularly on the tergites of the male, becomes apparent (Text-fig. 1, C, D).

The tergites and sternites of the abdomen may be removed, the underlying fat-body and muscles cleaned away and the remaining structures fixed and stained and mounted whole. In such preparations the distended glands in the tergites of the male appear highly active in the mature insect (Pl. 1 fig. 1). But the cell-bodies of many of the glands in the male and all of those



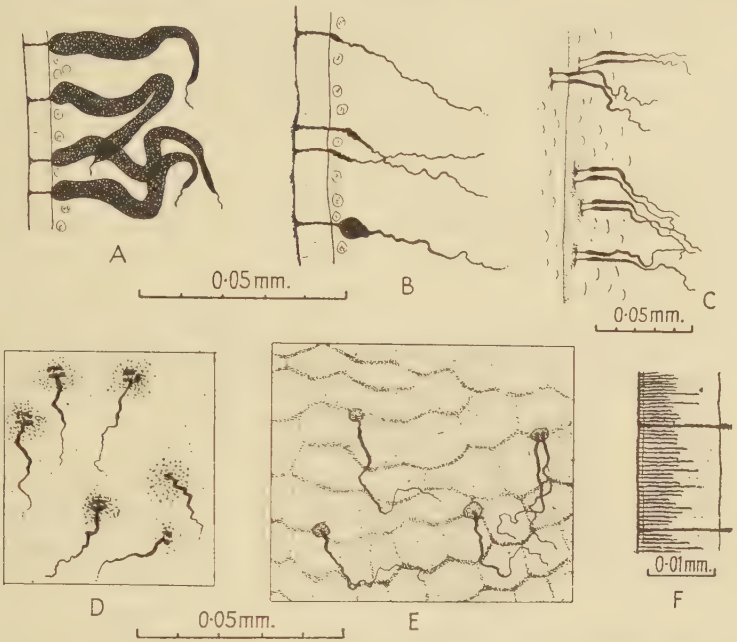
TEXT-FIG. 1. A, surface view of the anterior region of the seventh abdominal tergite in male *Periplaneta* showing the openings of the dermal glands. B, corresponding area in the female. C, surface view of part of the second abdominal tergite in male cockroach after treatment with boiling chloroform and ammoniacal silver hydroxide, showing distribution of dermal glands. D, corresponding area in the female.

in the female are completely shrunken so that the glands are almost impossible to detect without using the silver method. There are clearly two sorts of scattered dermal glands, one confined to the male and almost limited to the tergites, the other distributed over the tergites and sternites of both sexes.

Scattered dermal glands of the second type are responsible for secreting the cement layer of the epicuticle in *Tenebrio* and *Rhodnius* (Wigglesworth, 1947; Wigglesworth, 1948*a*). In these insects the cell-bodies of the glands are highly active and vacuolated during the last few days before moulting. At the time of moulting the elongated or sac-like receptacles of the glands are distended with secretion and the cell-bodies are in process of atrophy. At the moment of moulting or soon afterwards the gland contents are poured out to form the outermost covering of the epicuticle.

It has not been possible to follow the early stages in the deposition of the new cuticle in *Periplaneta*. The earliest stage at which the moulting insects

can be recognized is when they are distended with air and the old cuticle is just about to crack. If, at this stage, the new cuticle of a female *Periplaneta* is exposed, the insect boiled in chloroform for 5 minutes and then immersed in 5 per cent. ammoniacal silver hydroxide for 1 hour, the contents of the



TEXT-FIG. 2. A, female cockroach in the act of moulting; silver staining after boiling chloroform. Optical section of fully distended dermal glands at lateral margin of sternites. B, another part of the same specimen showing dermal glands emptied and silver staining material on surface of cuticle. C, surface view of part of male sternite 1½ hours after moulting; silver staining after chloroform. The thickened ridge of cuticle marks the limit of the area covered by the preceding segment. Along this ridge the emptied glands are largely in pairs. D, male cockroach just escaping from old cuticle, treated with ammoniacal silver hydroxide after ¼ minute in chloroform at room temperature; part of sixth abdominal sternite. Granular silver-staining contents of the glands in process of discharge from the mouths of the ducts. E, part of tergites of female cockroach 3 hours after moulting; silver staining after brief immersion in chloroform at room temperature. Glands emptied; cement appears as finely granular layer concentrated particularly in the sculptural folds of the cuticle. F, newly moulted cockroach; silver staining after boiling for 5 minutes in chloroform. Optical section of intersegmental fold in abdomen showing ducts of two dermal glands and pore canals stained with silver in their outer halves, penetrating the cuticulin layer to reach the surface.

dermal glands stain black. It is convenient to use the female because, as noted above, the linings of the innumerable glands of the male likewise stain with silver after chloroform extraction and they obscure the changes in the other glands. In some places the elongated gland vesicles are still distended with secretion and the silver-staining material which is the future cement layer is not present on the surface (Text-fig. 2A). In other places the gland vesicles are reduced to convoluted black threads and the blackened

contents of the glands may be seen on the surface of the cuticle (Text-fig. 2B).

In many places the glands are arranged in pairs so that the two ducts discharge side by side (Text-fig. 2C). It has not been possible to detect any difference in the staining properties of the contents of such adjacent glands. There is no reduction of silver in either gland of the pair if the insect is immersed in the ammoniacal silver solution without previous treatment with chloroform. It is not possible to claim, therefore, that one gland secretes protein and the other quinone—like the left and right accessory glands which form the oötheca (Pryor, 1940).

Other phenomena observed in *Tenebrio* (Wigglesworth, 1948*a*) can be confirmed in *Periplaneta*. After boiling in chloroform there may be a diffuse reduction of silver in the substance of the cuticle. There is commonly an intense reduction forming a hexagonal cap over the area of cuticle corresponding to each epidermal cell (the polyphenol layer). Sometimes the pore canals stain deeply with silver and are then seen to penetrate the cuticulin layer and become continuous with the silver staining 'polyphenol layer' (Text-fig. 2F). If the cockroach soon after moulting is treated very gently with chloroform (immersion at room temperature for half a minute or so) the cement layer appears as a finely granular deposit, spreading out from the mouths of the dermal glands (Text-fig. 2D). It probably forms a continuous thin sheet, but by filling in the depressions along the boundaries of the cell areas, it appears as a dark meshwork (Text-fig. 2E). Gradually the cement layer and the exocuticle harden and then, after treatment with chloroform, only the lining of the dermal glands will stain with the silver.

The histological picture is complicated by the large number of other dermal glands present in the male cockroach. Moreover, since it is not possible to recognize nymphs that are preparing to moult, it is not possible to observe the glands at known intervals before the old cuticle is shed. But there can be little doubt that a cement layer exists in the cockroach and that it is poured out by certain of the dermal glands at the time of moulting.

Glandular Activity in the Mature Insect; the Oenocytes and Wax Secretion

The waxy secretion of the cockroach is present in both sexes. It is a soft material, freely exposed on the surface of the cuticle, which must be readily removed by contact with the surroundings. Presumably it is secreted continuously in the mature insect. The dermal glands which produce the cement layer, as we have seen, are completely atrophied in the mature adult. The sac-like glands between the fifth and sixth abdominal tergites are usually regarded as stink glands. In some species they are present only in the male (Konček, 1924). The conspicuous dermal glands on the abdominal tergites of the male cannot be responsible for a secretion present in both sexes. It does not appear, therefore, that any of the dermal glands can be responsible for the secretion of the cuticular wax in the mature cockroach.

Dusham (1918) concluded that the wax came from the 'glandular cells' described by Minchin (1888) and Mingazzini (1889). These cells are now recognized as sub-epidermal oenocytes (Stanislavskij, 1926). It is characteristic of the oenocytes as described in detail in *Rhodnius* (Wigglesworth, 1933, 1947) and *Tenebrio* (Wigglesworth, 1948*a*) that during the moulting cycle they reach the peak of their secretory activity precisely at the moment when the 'cuticulin' layer of the epicuticle is being laid down. Cuticulin is apparently composed of lipoprotein. It was therefore concluded that the oenocytes are epidermal cells which have become specialized for the synthesis of lipoprotein, and that their renewed activity in the adult *Rhodnius*, which is much more evident in the female, is related with the deposition of similar components in the egg-shell (Wigglesworth, 1933) in which lipoproteins are an important constituent (Beament, 1946).

The nature of the lipoid component of cuticulin is not known. If it should prove to be a wax the ideas outlined above would tally with the conclusions of Hollande (1914) who, on the basis of the staining reactions and solubilities of the inclusions in these cells, believed the oenocytes to be concerned in wax metabolism. It is of interest to recall that the wax secreted by Géné's organ in the female tick *Ornithodorus* is apparently stored and mobilized in combination with protein (Lees and Beament, 1948). The functional similarity of wax secretion and cuticulin secretion is therefore very close.

As described by Dusham (1918) the oenocytes of the cockroach are distributed below the epidermis of the abdominal tergites and sternites, particularly over the anterior half of each segment where it is overlapped by the segment before. They are extremely abundant and conspicuous; far more so than in *Rhodnius* or *Tenebrio*. It is reasonable to correlate this with the fact that the cockroach is secreting wax continuously throughout its life.

But it is desirable to obtain some direct evidence that the oenocytes are producing this wax. They contain variable quantities of an amber-coloured pigment in the form of refractile spheres and granules, but there is no reason to suppose that this represents their chief secretory product. The staining reactions of the oenocytes of the cockroach are the same as those of *Rhodnius* and *Tenebrio*. With Delafield's haematoxylin the cytoplasm stains pink with a finely dispersed blue granular meshwork. With osmium tetroxide (Altmann's fixative) they stain varying shades of grey. And after fixation with Bouin's fluid and staining with the fat stain BZL blue (Ciba), counterstained with Bismarck brown, they show a diffuse blue of varying intensity.

It has already been shown that the soft wax of the cockroach is sufficiently mobile at room temperature to be adsorbed by alumina dust (Beament, 1945; Wigglesworth, 1945). This observation suggested the possibility of inducing differential secretory activity in the wax-producing organs on the two sides of the body—in the hope that there might be visible differences in the oenocytes or some other structure.

Mature female cockroaches were held down by securing the wings in a spring clip mounted in plasticine. The hind legs were likewise held down by

means of plasticine which also prevented the movement of the abdomen from side to side. Finally, a microscope slide, mounted on edge in plasticine, was applied as a partition along the mid-line of the abdomen, holding this firmly against the ground. A layer of alumina about 2 mm. thick was then applied to the right half of the abdomen. The insects were kept well supplied with sugar and water placed on absorbent paper below the head; they were dissected after periods of 1 to 7 days.

The tergites were removed, stretched on pins, fixed with Bouin's fluid, and the longitudinal muscles carefully removed without injury to the underlying oenocytes. They were then stained in haematoxylin and mounted entire with the cuticle uppermost. A few specimens were stained in BZL blue, and a few were fixed in Altmann's fluid.

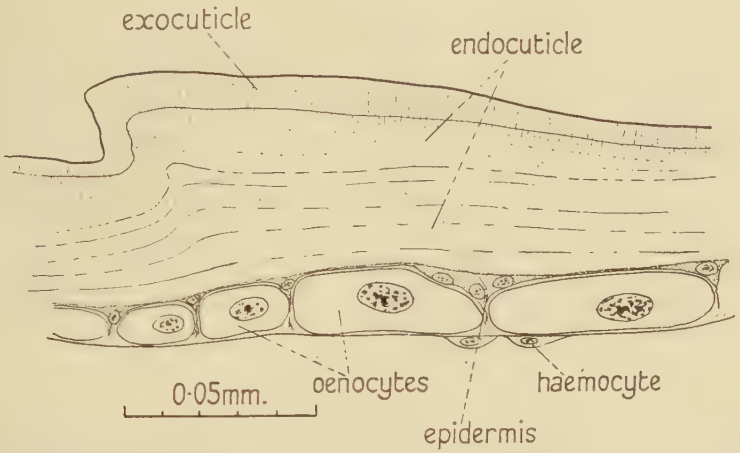
Twelve insects were treated in this way. No difference between the two sides could be detected in any structure with the exception of the oenocytes. The appearance of these varied considerably, but when corresponding areas on the two sides were compared, slight consistent differences could be seen at all times from 24 hours onwards. On the control side the cells were clear and distended. (Pl. 1, figs. 3 and 5.) On the side on which the alumina dust had rested, particularly in the exposed areas unprotected by the preceding segment, they were usually more shrunken, the cytoplasm was more deeply staining, and their lobes sometimes vacuolated, sometimes darkly staining and apparently breaking up (Pl. 1, figs. 4 and 6). In specimens stained with osmium tetroxide (Altmann's fluid) the oenocytes on the side exposed to alumina were more contracted and stained in general a deeper grey. Similarly, in specimens stained with BZL blue, the oenocytes on the treated side were more contracted and stained more deeply.

All these changes are compatible with an increased secretory activity by the oenocytes. In the insects used there was always abundant haemolymph; the observed effects cannot be the result of differential desiccation on the two sides.

If the oenocytes are producing the cuticular wax the question arises, how it is conveyed to the surface of the cuticle. It is characteristic of the wax glands of the bee (Dreyling, 1906), of *Aleurodes* (Weber, 1935), and other insects that the wax is conveyed from the secretory cells, not by way of special ducts, but through pore canals which traverse the general substance of the cuticle. Dusham (1918) claimed that this was the case in the cockroach.

There is evidence that the pore canals in *Rhodnius* (Wigglesworth, 1947) and *Tenebrio* (Wigglesworth, 1948*a*) by which the cuticular wax is conveyed to the surface, penetrate the cuticulin layer of the epicuticle. In *Rhodnius* and other insects (Wigglesworth, 1945) and in the tick *Ornithodorus* (Lees, 1947), if the cement and wax layers are injured by abrasion, fresh wax is secreted through the cuticle to repair the damage. In *Periplaneta* it is claimed by Richards and Anderson (1942) that the pore canals (as revealed by the electron microscope) do not penetrate within 2 μ of the surface of the epicuticle. But we have seen that in the newly moulted cockroach the silver-staining

filaments may continue from the pore canals right to the surface. And in sections of the abdominal cuticle in the mature cockroach deeply stained with iron haematoxylin the pore canals will sometimes take up the stain conspicuously and then it is possible to follow them in places, with the light microscope, apparently right up to the limiting membrane provided by the cement layer. On the other hand, there is no proof that the waxes are actually passing out through the pore canals. As shown by Way (1949), in the caterpillar *Diataraxia*, wax is secreted through cuticle in which pore canals are lacking.



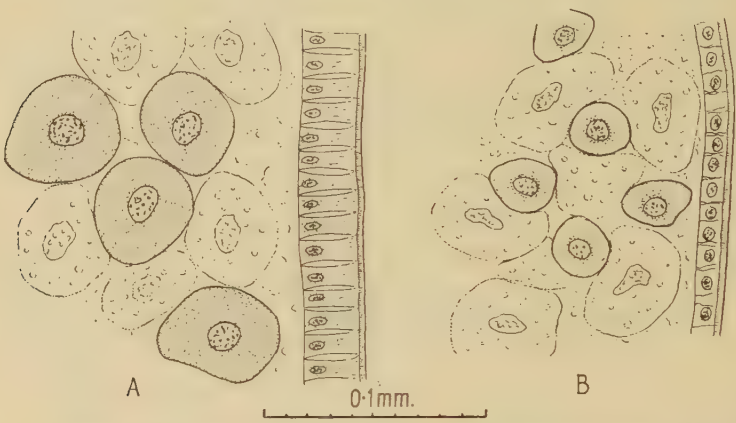
Text-fig. 3. Longitudinal section of tergites in mature female cockroach. The ridge marks the limit of the area covered by the preceding segment.

During the formation of the cuticle the epidermal cells are large and evenly dispersed in a hexagonal pattern below the cuticle into which the pore canals run. In the mature cockroach, the epidermal cells are much attenuated and their nuclei displaced by the enlarged oenocytes which come into close contact with the cuticle. Stanislavskij (1926) figures certain of the oenocytes lying in direct contact with the cuticle. If that were so perhaps they might discharge their wax secretion directly into the pore canals or into the substance of the cuticle. But in the best of our sections, even where the nuclei of the epidermal cells are greatly displaced, an excessively thin cytoplasmic membrane belonging to the epidermal cells appears to separate the oenocytes from the cuticle (Text-fig. 3). One must suppose, therefore, that the attenuated epidermal cells act as intermediaries in the transmission of the wax to the surface.

Dusham (1918) supported his contention that the wax is discharged through the pore canals by reference to the wax glands of the bee in which this is certainly the case. In the wax-secreting bee the epidermal cells concerned are greatly enlarged to form a high columnar epithelium richly supplied with tracheae; only in the foraging bee which has ceased to produce wax do the

epidermal cells become reduced in size. It was interesting to see whether there are differences between the oenocytes of the wax-secreting bee and the foraging bee.

The oenocytes of the bee are dispersed among the cells of the fat-body which they resemble rather closely in histological sections. In unstained preparations, whether mounted whole or in sections, the oenocytes are readily distinguished by their yellowish colour and more refractile cytoplasm. It can then be seen that they are considerably larger in the bee at the height of wax secretion than they are in the foraging bee (Text-fig. 4, A, B). And in



Text-fig. 4. Sections of the wax-secreting epidermis and overlying cuticle of the honey-bee and the underlying fat-body cells and oenocytes. For the sake of clearness the fat-body cells are only faintly indicated. A, wax-secreting bee; oenocytes and wax-gland epithelium enlarged. B, foraging bee; oenocytes and wax-gland epithelium reduced.

sections stained with haematoxylin the cytoplasm of the oenocytes stains deeply throughout in the wax-secreting bee; whereas in the foraging bee the cytoplasm is rather pale staining except around the nucleus. This evidence does not take us very far; but it is clear that at the time when the wax-secreting epidermal cells of the bee are most active, the oenocytes also show signs of greatest activity.

Discussion

As a first approximation the epicuticle of the insect was described as consisting of superimposed layers (Wigglesworth, 1947). But it has been shown that if the cement and wax layers are damaged by abrasion fresh wax can be secreted by the epidermal cells through the substance of the cuticle (in *Rhodnius*, *Pieris* pupa, &c., Wigglesworth, 1945, and in the tick *Ornithodorus*, Lees, 1947). In the organ of Géné, by means of which the female *Ornithodorus* secretes wax over the surface of the eggs, there is evidence that a cement layer is lacking (Lees and Beament, 1948). But the wax glands of the bee, and the cuticle of the cockroach as described in this paper, demonstrate clearly enough that it is possible for the insect to pass its wax out even through

a cement layer covering a more or less sclerotized cuticle. This raises the question whether, for some insects, the laminar picture of the epicuticle may not be too schematic and whether the layers may not be impregnated to a varying extent by the diffusing waxes. In the egg of *Ornithodorus*, Lees and Beament (1948) adduce evidence that the waterproofing wax, at first wholly superficial, gradually soaks into the substance of the shell. And the inert serosal membrane of the *Rhodnius* egg becomes impregnated with wax secreted by the developing ovum (Beament, 1949).

It appears that the oenocytes are the most probable source of the cuticular wax in the cockroach. Three functions (among others) have been suggested for the oenocytes of insects. (i) That they are concerned in wax metabolism (Hollande, 1914). (ii) That they secrete the cuticular wax in the cockroach (Dusham, 1918, and the present authors). (iii) That they are concerned in the production of the lipoproteins which compose the cuticulin layer of the epicuticle and some parts of the egg-shell (Wigglesworth, 1933, 1947, 1948*a*). The chemical nature of the lipoid component of cuticulin is not known. If it should prove to be a wax, all three views would fall into line.

We are indebted to Dr. V. Novak of the University of Prague for the translation of the paper by Stanislavskij.

Summary

In the epicuticle of the cockroach there is a cement layer formed, as in other insects, by secretion from certain of the dermal glands at the time of moulting. These dermal glands are widely distributed over the abdominal tergites and sternites in both sexes. Their cell-bodies are atrophied in the mature insect. They differ in this respect from the very numerous dermal glands on the abdominal tergites of the male, which remain distended with vacuoles.

The cuticular wax, which is freely exposed on the surface of the cuticle, is thought to be produced by the sub-epidermal oenocytes and to be discharged during the life of the cockroach, perhaps through the pore canals, by the epidermal cells.

The oenocytes of the honey-bee are much larger during the height of wax secretion than they are in the foraging bee.

The view that the oenocytes are concerned in wax metabolism is compatible with the view that they synthesize the lipoprotein (or wax protein) components of the cuticle and egg-shell.

REFERENCES

BEAMENT, J. W. L., 1945. J. exp. Biol., **21,** 115.
—— 1946. Quart. J. micr. Sci., **87,** 393.
—— 1949. Bull. ent. Res., **39,** 467.
DREYLING, L., 1906. Zool. Jahrb., Abt. Anat., **22,** 289.
DUSHAM, E. H., 1918. J. Morph., **31,** 563.
HOLLANDE, A. C., 1914. Arch. Anat. Microsc., **16,** 1.
KONČEK, S. K., 1924. Z. wiss. Zool., **122,** 311.
LEES, A. D., 1947. J. exp. Biol., **23,** 379.
—— and BEAMENT, J. W. L., 1948. Quart. J. micr. Sci., **89,** 291.
MINCHIN, E. A., 1888. Ibid. **29,** 229.
MINGAZZINI, P., 1889. R.C. Accad. Lincei, **5,** 573.
PRYOR, M. G. M., 1940. Proc. Roy. Soc. B, **128,** 378.
RAMSAY, J. A., 1935. J. exp. Biol., **12,** 373.
RICHARDS, A. G., and ANDERSON, T. F., 1942. J. Morph., **71,** 135.
STANISLAVSKIJ, B., 1926. Pub. Fac. Sci. Univ. Charles, **56,** 1.
WAY, M. J., 1949. Quart. J. micr. Sci. (In the press.)
WEBER, H., 1935. Zoologica, **33,** 1.
WIGGLESWORTH, V. B., 1933. Quart. J. micr. Sci., **76,** 269.
—— 1945. J. exp. Biol., **21,** 97.
—— 1947. Proc. Roy. Soc. B., **134,** 163.
—— 1948*a*. Quart. J. micr. Sci., **89,** 197.
—— 1948*b*. Biol. Rev., **23,** 408.

DESCRIPTION OF PLATE I

All figures represent whole mounts of the cuticle and underlying structures stained with Delafield's haematoxylin and photographed in surface view with a 4-mm. objective.

Fig. 1. Epidermis and associated structures in the tergites of male *Periplaneta*. The numerous dermal glands are clear and distended, the oenocytes chiefly in pairs.

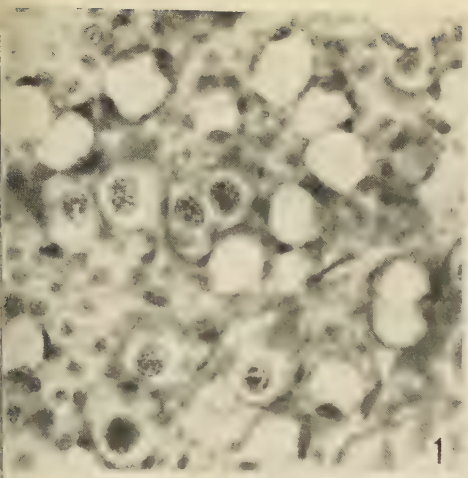
Fig. 2. The same in tergites of female. The nuclei of the epidermal cells are displaced by the crowded oenocytes. No dermal glands are visible.

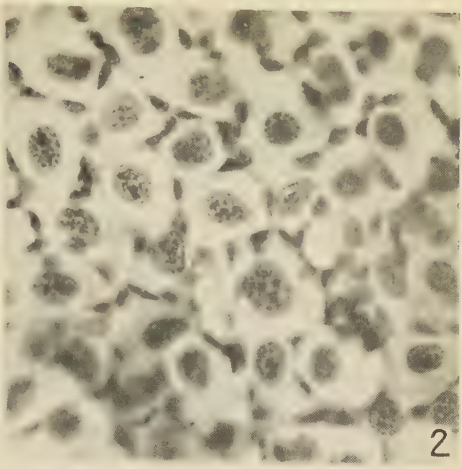
Fig. 3. Oenocytes below tergites of female *Periplaneta*, normal side: oenocytes clear and distended.

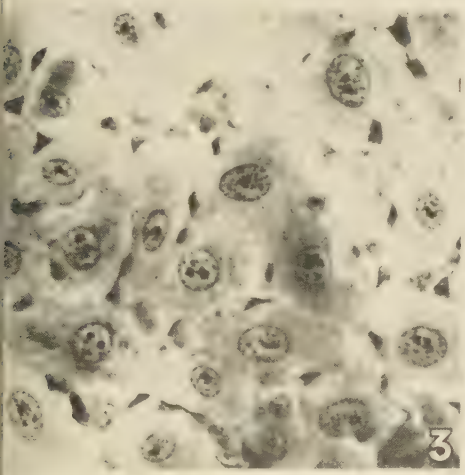
Fig. 4. Oenocytes below tergites of the same insect as fig. 3, in the corresponding area of the side in contact with alumina for 2 days: oenocytes shrunken and vacuolated.

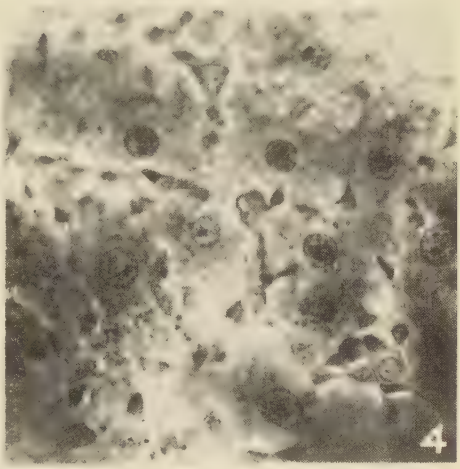
Fig. 5. Oenocytes below tergites of female, normal side: cells clear and distended.

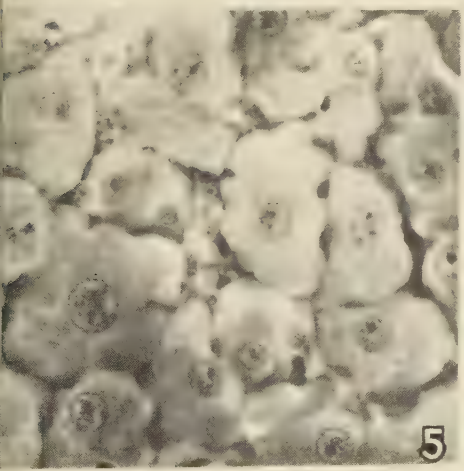
Fig. 6. Oenocytes below tergites of the same insect as fig. 5, in the corresponding area of the side in contact with alumina for 5 days: oenocytes darkly staining and shrunken.

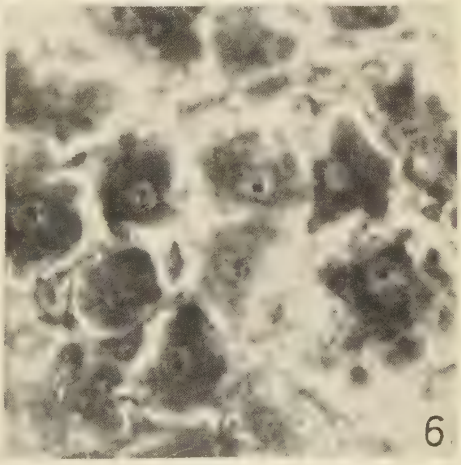












S. KRAMER AND V. B. WIGGLESWORTH.—PLATE I

A Study of the Male Germ-cells of the Rat and the Mouse by Phase-contrast Microscopy

BY

R. A. R. GRESSON

(*From the Department of Zoology, University of Edinburgh*)

With one Plate

Introduction

THE cytoplasmic structures of the male germ-cells of mammals and the metamorphosis of the mammalian spermatid have been studied extensively. As the stages of spermateleosis are essentially similar in all the mammals investigated, little remains to be done with the ordinary light microscope, except to determine variations in minor detail exhibited by different animals, and perhaps to examine certain cell structures in particularly favourable material. While it is true that further advances in our knowledge of the male germ-cell must be based mainly on investigation of its submicroscopic structure, and on an understanding of the chemical nature and functions of its various parts, information on certain of the morphological features of the spermatid and of the sperm is still incomplete. Knowledge of the Golgi material and of the other cytoplasmic structures has been gained by the examination of material prepared by special methods and it is possible that these methods do not give a true representation of the living cell. The introduction of phase-contrast microscopy has made possible the examination of many of the minute structures of the living cell, and so enables comparison to be made between the picture presented by fixed preparations and the conditions in living material.

The writer has previously studied the spermatogenesis of the mouse (Gresson, 1942) and the rat (Gresson and Zlotnik, 1945). The present work with the phase-contrast microscope was undertaken in an attempt to identify and examine the various cytoplasmic bodies previously seen in fixed and stained preparations of the testes of these animals.

Material and Methods

Small pieces of the testes were teased and mounted in normal saline. It was found desirable to exert gentle pressure on the cover-glass so as slightly to flatten the cells. The cover-glass was sealed with a thin smear of vaseline. The cells prepared for examination in this way usually retained a normal appearance for about 1 hour, but the preparations were generally discarded after about half an hour. A Cooke phase-contrast equipment was used. Difficulties

were experienced in obtaining photomicrographs with a 2-mm. oil immersion objective; consequently the photomicrographs were taken with a 4-mm. objective and subsequently enlarged.

I wish to thank Professor James Ritchie, C.B.E., for research facilities and Mr. N. Macdonald, Senior Technician, for taking the photomicrographs. The work was aided by a grant from the Earl of Moray Endowment of the University of Edinburgh.

Observations

Spermatogenesis of the Rat

Primary Spermatocytes. In non-dividing cells the mitochondria are invariably seen as dark granules, some of which are often arranged in short rows and small clumps. The majority of the mitochondria of the young spermatocyte are concentrated at one pole of the nucleus, and at a later stage are distributed laterally to the nucleus, thus leaving the poles of the cell relatively free of granules. Prior to the first meiotic division the mitochondria are fairly evenly scattered throughout the cell.

The Golgi material was clearly seen in a very large number of the primary spermatocytes examined. It consists of slender dark rods and filaments which surround a spherical area at one pole of the nucleus.

Secondary Spermatocytes. The mitochondria and Golgi elements are similar to those of the primary spermatocytes. The Golgi material is in most cases more clearly visible (Pl. 1, fig. 3).

Spermatids. The mitochondria of the young spermatid are granular; they are scattered through the cytoplasm but tend to form small clumps. They were observed throughout the stages of spermateleosis, and in the late spermatid are present in the region posterior to the nucleus, where they appear to be slightly larger than those of the younger cells.

The Golgi material of the young spermatid lies at one pole of the nucleus and is composed of rods and short filaments. Although the Golgi elements are shorter than those of the spermatocytes they are usually darker and more easily seen. They form a fairly compact mass, but in some cells a clear central area is visible (Pl. 1, fig. 5). As the archoplasmic vacuole increases in size the Golgi material moves to the vicinity of the distal pole of the nucleus, and there separates into two parts. The smaller portion remains close to the nucleus and the other part forms the Golgi remnant (Pl. 1, fig. 6). Each part consists of a dark external region and a lighter central area.

When the Golgi material has moved away from the anterior pole of the nucleus, a large body, closely resembling the Golgi material but situated some distance from it, was very frequently observed. This body appears to be the structure previously identified in spermatids of the rat and then named the residual body (Gresson and Zlotnik, 1945).

The archoplasmic vacuole, the proacrosome and the stages of the development of the acrosome were observed. The proximal and distal centrioles and the axial filament were seen in a number of cells. A clear spherical body

ossessing a slender dark outline was occasionally visible in the neighbourood of the distal pole of the nucleus of the late spermatid. It is difficult to onnect this body with any of the structures observed in fixed material; it is uggested, however, that it may be an accessory body. The manchette was isible at a late stage of spermateleosis.

Spermatozoa. Examination of spermatozoa revealed the presence of the crosome and of a small dark area of the head immediately adjacent to the eck. The dark area is the post-nuclear cap. The granules of the mitohondrial sheath were visible and in many cases appeared to be arranged in a piral manner around the axial filament. Frequently, however, the granules ere very closely packed together and consequently the spiral was not aparent. The ring centriole was often seen at the posterior end of the middleiece. Careful examination of the neck sometimes showed that a dark granule as present close to the head. It was not determined if the granule was the roximal centriole or the accessory body.

Spermatozoa in freshly prepared smears of the testes possessed a protolasmic bead which is usually situated in the neck region, but may be present nore posteriorly on the middle-piece. Small spherical bodies or granules ere often observed within the protoplasmic bead. In preparations which ad been kept longer, many of the sperms lacked a protoplasmic bead, sugesting that this structure tends to be lost in spermatozoa kept in normal aline.

Spermatogenesis of the Mouse

Primary Spermatocytes. The Golgi material was invisible in the majority f the primary spermatocytes examined. In many cells, however, a clear area ree of mitochondria was seen at one pole of the nucleus. In some cases this rea was not in contact with the nuclear membrane, while in others it appeared o extend over a small part of the nucleus. In position and roughly in size the lear area resembled the region occupied by the Golgi material in osmic and n silver preparations. On careful examination a few slender rods and granules ere occasionally seen surrounding the clear area; it is probable that they were Golgi elements. The mitochondria resembled those of the rat (Pl. 1, figs. 1 nd 2).

A few primary spermatocytes were observed to be in division. In these ells granular mitochondria were scattered through the cytoplasm. Golgi lements were not identified.

Secondary Spermatocytes. The mitochondria and Golgi elements were imilar to those of the primary spermatocytes. In many cells two or three mall spherical bodies with dark rims were present; in some cases two such odies were in contact. They resembled the spherical bodies observed in the ate spermatids of the rat.

Spermatids. The Golgi material of the spermatid, unlike that of the spermatocytes, was usually very clearly seen as a body which is much darker han the surrounding cytoplasm (Pl. 1, fig. 7). The examination of the Golgi

material, mitochondria, archoplasmic vacuole, proacrosome, and developi
acrosome confirmed previous observations on fixed material (Pl. 1, fig. 4)..

The proximal and distal centrioles and the axial filament were frequen
visible. A clear spherical body, similar to that observed in the rat, w
occasionally present in late spermatids. The manchette was not seen.

Spermatozoa. The structures seen in the spermatozoa of the rat are visib
in those of the mouse.

Discussion

Most of the structures previously studied in fixed preparations (Gressc
1942; Gresson and Zlotnik, 1945) were identified with the phase-contra
microscope. The present studies, therefore, show that good osmic and silv
preparations, such as Flemming (without acetic acid), Champy-Kull, Kola
chev, Da Fano, and Aoyama, present a picture of the various cytoplasm
components which closely resembles the structure of the living cell.

In form and distribution, the mitochondria seen with the phase-contra
microscope very closely resemble those of fixed and stained sections, exce
that in fresh smears of living material they are more numerous at the extrem
periphery of the primary and secondary spermatocytes. In preparatio
which were under examination for slightly more than 1 hour the mit
chondria tend to become concentrated in the cytoplasm adjacent to th
nucleus, and frequently to increase slightly in size.

Examination of the localized Golgi material of spermatocytes and spermati
indicates that it is composed of rods and filaments which do not form a tru
network. This agrees with previous work on the mouse (Gresson, 1942) an
on the rat, except that in the latter Gresson and Zlotnik (1945) believed th
the localized Golgi material was made up of rods and granules.

Price, Jones, and Smyth (1946), by phase-contrast microscopy, identifie
the Golgi material of the spermatid of the mouse as a dark crescent resting c
a clear area in contact with the nucleus. The writer found that the Gol
elements of the spermatid were often closely packed together, but in oth
cases they were seen to surround a clear crescent. Oettlé (1948) identifie
the Golgi material of the human primary spermatocyte as a dark crescent i
which a cortex and a medulla were sometimes visible. He observed a simila
structure in the spermatid but not in the secondary spermatocyte. In th
late spermatid the Golgi material was present distal to the nucleus.

Oettlé states that rodlets were present outside the spindle of dividin
primary spermatocytes, but these could not definitely be identified as Golg
bodies. The present writer examined dividing cells but failed to see Golg
bodies.

It is of interest that the Golgi bodies of the spermatocytes of the rat wer
much more easily seen than those of the mouse. In the mouse the Golg
material was more clearly shown in the secondary spermatocytes than in th
primary spermatocytes, and in the spermatids was visible as a conspicuou
dark body. A similar progressive darkening was observed in the cells of th

t. It would appear, therefore, that differences exist between the Golgi aterial of the spermatocytes of the two animals, and that some change takes lace in the secondary spermatocytes and in the spermatids which render the aterial more easily visible.

The structures seen in the spermatid during its metamorphosis resemble ose of fixed material, and need not be discussed here. It may be mentioned, owever, that the manchette was faintly visible in late spermatids of the rat ut not in those of the mouse, and that the present work supports the view at the distal centriole does not divide. Oettlé claims that the manchette, e acrosome and the archoplasmic vacuole are visible in the human spermatid, d that a body which is probably a post-nuclear body is sometimes present. e figures mitochondria but does not describe them.

Price, Jones, and Smyth, in a short note, state that they failed to identify e acrosome of the mouse, but that otherwise 'the cytological picture' of the ermatid is similar to that figured by the writer (Gresson, 1942) from olatchev preparations.

Summary

1. The cytoplasmic components of the male germ-cells of the rat and the ouse were examined by phase-contrast microscopy. Most of the structures en in fixed and stained preparations were identified.

2. The localized Golgi material of spermatocytes and spermatids consists f rods and filaments. The mitochondria are granular.

3. The archoplasmic vacuole, proacrosome, developing acrosome, proxi-al and distal centrioles, and the axial filament were visible. A structure hich may be an accessory body was observed in spermatocytes and spermatids. he manchette was visible in late spermatids of the rat but not in those of the ouse.

4. Examination of spermatozoa revealed the acrosome, the post-nuclear ap, the mitochondrial sheath, the ring centriole, and the protoplasmic bead. granule was sometimes visible in the neck region.

REFERENCES

RESSON, R. A. R., 1942. Proc. Roy. Soc. Edin. B, **61**, 197.
— and ZLOTNIK, I., 1945. Ibid., **62**, 137.
ETTLÉ, A. G., 1948. Nature, **162**, 76.
RICE, A. T., JONES, R., and SMYTH, J. D., 1946. Ibid., **157**, 553.

DESCRIPTION OF PLATE I

Photomicrographs taken with Cooke, Troughton and Sims phase-contrast equipment.

Figs. 1 and 2. Primary spermatocytes of mouse showing mitochondria.
Fig. 3. Secondary spermatocytes of rat to show Golgi material.
Fig. 4. Spermatids of mouse. The proacrosome, archoplasmic vacuole, and Golgi material re shown.

Fig. 5. Spermatid of rat to show Golgi material.

Fig. 6. Late spermatids of rat. The acrosome of each cell is shown. The Golgi mate and Golgi remnant of one cell are shown. Pressure was applied to the cover-glass and preparation was allowed to stand for about 1 hour. The cells are flatter than in the ot preparations, and the mitochondria are somewhat swollen. The other structures do not sh degenerative changes.

Fig. 7. Young spermatids of mouse. The Golgi material is shown in most of the cells.

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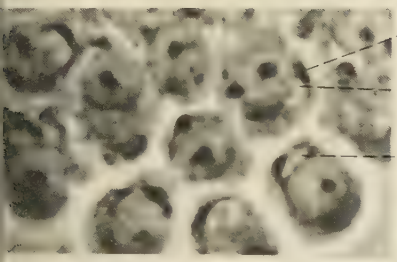

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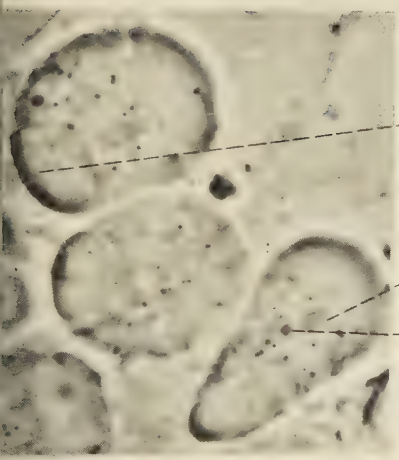

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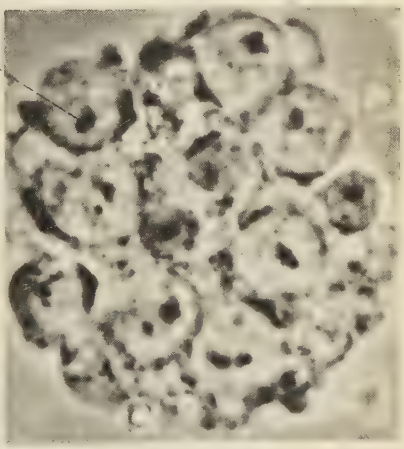


7

R. A. R. GRESSON.—PLATE I

Cytochemical Studies on the Embryonic Development of *Drosophila melanogaster*

II. Alkaline and Acid Phosphatases

BY

T. YAO

(*From the Institute of Animal Genetics, Edinburgh; present address, Dept. of Biology, National University of Che-Kiang, Hangchow, China*)

With two Plates

THE distribution of phosphatases during the embryonic development of *Drosophila melanogaster* Meig. was chosen as the next object of study for three main reasons: (1) a satisfactory technique for the demonstration of phosphatase (at least in the case of alkaline phosphatase) is available; (2) the possible significance of phosphatases during embryogenesis is indicated by the work of Moog (1944); and (3) the *Drosophila* embryo provides favourable material for a study of the relation between the phosphatases on one hand and growth and differentiation on the other, since a dissociation of these two morphogenetic processes is evident (Yao, 1950).

Material and Methods

The material and the procedures designed to obtain the requisite stages of the developing oocytes and embryos have been described in the first paper of this series.

For the demonstration of alkaline phosphatase, Danielli's (1946) method was generally adhered to. Certain minor modifications in timing were, however, found to be necessary for the present material, because of the relatively low content of the enzyme. Eighty per cent. alcohol was used as fixative. The duration of the steps between fixation and incubation was therefore deliberately shortened, especially that in the paraffin bath (m.-p. 52° C., 1–1½ hours at 56° C.). Sections were cut at 10μ. Sodium glycerophosphate (B.D.H.), consisting of almost pure beta-salt, was used as substrate. The pH of the incubation mixture was 9·40. Since it was found that the alkaline phosphatase of *Drosophila* is also activated by Mg ion, magnesium chloride was added to the medium to give a final concentration of 0·01 M. The duration of incubation was 4 hours at 37·5° C. In several cases, the period was prolonged to 8, 12, and 24 hours.

Gomori's technique for the demonstration of acid phosphatase has recently been questioned on certain grounds (Bartelmez and Bensley, 1947, and others). The problem of non-specific adsorption was therefore studied, using *Droso-*

phila ovaries, by superimposing active sections on inactivated (5–10 minutes in boiling water) sections and incubating them together for various lengths of time (8, 12, 24, 48 and 72 hours). It was found that after short incubation (8–12 hours) the inactivated sections were practically unstained. Non-specific diffuse yellowish staining in these sections became evident in 24-hour series when the active sections gave a dark golden-brown reaction. Such contamination was more intense after 48- and 72-hour incubation. From this result, it appears that incubation requires very critical timing so as to give a maximal possible reaction but at the same time a minimum of non-specific complications. Moreover, lead phosphate was found to be rather stable, for alternate sections of ovaries show almost no difference in the reaction intensity whether they are immediately visualized after incubation or visualized after a preliminary soaking in distilled water for 24 hours subsequent to incubation. On the other hand, lead sulphide was found to be extractable by distilled water: soaking for a few hours can already produce a visible effect. Furthermore, the distribution of acid phosphatase in the *Drosophila* ovary has been checked by incubating fresh unfixed material: enzyme localization, identical to that found in the fixed sections, was obtained: this will be mentioned later.

Moog's (1944) modified procedure for beta-glycerophosphate was used in this work. Chilled acetone was used as fixative throughout (80 per cent. alcohol destroyed nearly all the acid phosphatase activity in *Drosophila* material). The pH of the resulting incubation mixture as determined potentiometrically was 4·70. Incubation at pH 5·0 and 5·3 was also tried. However, owing to the heavy precipitation of beta-glycerophosphate under these conditions, detailed study was not possible. The duration of incubation was 16 hours at 37·5° C. Control sections were incubated either without substrate or in the presence of 0·01 M. fluoride.

The difficulties which other workers have experienced in the histochemical demonstration of acid phosphatase have also been encountered in the present material. Evidently this is mainly due to the fact that both fixation and paraffin embedding cause a far greater loss of activity for acid phosphatase than for alkaline phosphatase (Stafford and Atkinson, 1948). An effort was made to intensify the reaction by adding l-ascorbic acid to the incubation mixture (Moog, 1944). About a fourfold increase of enzyme activity was obtained with a concentration of ascorbic acid as low as 0·005 M. The above value was estimated from the difference in the time of incubation required for attaining an approximately equal intensity of enzyme reaction in the presence and in the absence of ascorbic acid. However, the use of ascorbic acid was complicated by the formation of yellowish-white turbidity in the medium. The results to be described in this paper are based on reactions carried out without the addition of l-ascorbic acid.

The localization of phosphatases in *Drosophila* testes has also been studied: the result will be briefly dealt with.

Results

I. *Alkaline Phosphatase*

(*a*) *Oocyte development.* Alkaline phosphatase is not demonstrable in *Drosophila* ovaries taken from 1-day-old females. Even when incubation was extended to 24 or 48 hours, only a very feeble reaction could be noticed in the nucleolar materials of the nurse cells and follicular epithelial cells. Both experimental and control sections appear greyish, possibly due to the presence of preformed phosphate. However, the epithelium of the oviducts gives a moderate to strong reaction.

Testes from adult males also show no histochemical trace of the enzyme.

(*b*) *Embryonic development.* Following on the negative reaction of mature oocyte and sperm, the enzyme is not detectable (even in the nuclei) in fertilized eggs nor in embryos up to a stage just prior to the contraction of the germ band. Alkaline phosphatase arises suddenly in the embryo during, or more likely immediately after, this morphogenetic stage. It invariably makes a first appearance in the ventral part of the embryo near the future thorax, although the exact centre of origin is rather difficult to locate. However, from a study of a great number of $8\frac{1}{2}$- to 9-hour embryos, I got the impression that the centre lies probably in the latero-ventral ectoderm (opposite the ventral nerve cord) of the metathoracic and first two abdominal segments. Topographically, these segments occupy almost a central position along the antero-posterior axis of the embryo and their hypodermis always show a stronger alkaline phosphatase reaction than does the hypodermis of other segments in the period soon after the appearance of enzyme activity (Pl. 1, fig. 1). In most cases, only one such centre is found on the one side of the median axis. Occasionally two centres, one on either side of the median axis, are found.

From the centre (or centres) the enzyme activity spreads to the neighbouring hypodermal cells and to the interior organs in a quite characteristic manner, for definite gradients of alkaline phosphatase reaction are always observable in embryos fixed between the 9th and 12th hour after laying. At least two such gradients are noticeable:

1. Extero-interior gradient. This is indicated by (i) the hypodermis always showing more enzyme activity than internal organs; (ii) the ventral nerve cord being at first active in its ventral part only; later there is a distinct gradient with greater activity in the ventral region; (iii) the mesodermal components of the intestine invariably reacting earlier than the intestinal cells themselves to the wave of enzyme spread.

2. Antero-posterior gradient. The posterior part of the embryo is more reactive than the anterior part, a characteristic which can be very well seen in 10- to 11-hour embryos. This gradation also manifests itself within the hypodermis. In the case of the proctodaeum, the highest enzyme activity is found near the anal opening, gradually fading away towards the midgut. These two gradients of alkaline phosphatase reaction can both be seen in Pl. 1, fig. 2.

The possibility that gradient activities might be artifacts of some kind ha been ruled out by the following experiment: a part of the enzyme activity c a number of fixed 11- to 12-hour embryos was destroyed individually by th local application of heat from a micro-cautery needle before embedding: th inactive areas in such embryos always gave a negative reaction irrespective c the positive reaction of the uninjured regions and of the duration of incuba tion. In other words, diffusion which might otherwise be the disturbing facto does not take place within the sections under experimental conditions. Con sequently, the observed gradients must be attributed to the differential rate of spreading of enzyme activity.

Further spreading of enzyme activity takes place between the 12th and 14t hour, mainly in the anterior and central parts of the embryo. At this time th gradients of alkaline phosphatase reaction are no longer very distinct. In fact the antero-posterior gradient is even replaced by a gradient in the revers direction in 12- to 13-hour embryos. This is apparently due to the fact tha organs in the anterior part of the embryo (such as the brain, stomodaeum cephalopharyngeal apparatus, and head mesoderm) have reached their maxi mal enzyme activity, while the activity in the posterior part of the embryo i already in decline. Because the highest enzyme activity of a particular orga seems to be maintained for only a short period, it is difficult to say at whic stage the *Drosophila* embryo, as a whole, has its maximal alkaline phosphatase content. It may be stated that all embryonic tissues show a positive reaction in 12- to 15-hour embryos (Pl. 1, fig. 3).

Alkaline phosphatase is more concentrated in nuclei, presumably in the chromatin materials. In the cytoplasm, a diffuse and moderate reaction is demonstrable in every organ between the 12th and 15th hour. When a cel begins to acquire phosphatase activity, the nucleus seems to be the first regio to become active. Activity in the cytoplasm comes later.

At about the 15th hour, enzyme activity starts to decrease in most tissues especially in the cytoplasm. Cells of the tracheal tubes, hypodermis, and cephalopharyngeal apparatus still retain their full activity, with perhaps even more activity in their cytoplasm. It is noteworthy that these three organs have a common physiological function, i.e. the secretion of cuticle.

Except in the case of the salivary glands, the organs in the anterior part of the embryo only give some residual nuclear activity (after 24-hour incubation) at the time of hatching. These organs include the nervous system, cephalo-pharyngeal apparatus, fore-gut, proventriculus, gastric caeca, fat bodies, muscles, and hypodermis. On the other hand, the posterior part of the embryo is more active, due mainly to the presence of the reactive mid- and hind-guts (Pl. 1, fig. 4). A localization of alkaline phosphatase in the striated border of the intestinal cells and a gradient of activity between the border and the cell nucleus has been observed. The phosphatase reaction of the tracheae, fat bodies, muscles, and gonads in the posterior half of the embryo is also very weak. Posterior branches of the Malpighian tubes are, however, moderately active.

No alkaline phosphatase activity has been found in the yolk materials during most of the embryonic period. In older embryos (16- to 18-hour) the disintegrating yolk granules in certain sections of the mid-gut are reactive. Since in these cases the gut epithelia and its striated border always show a very high phosphatase content, the enzyme activity found in the yolk is probably not of intrinsic origin. Yolk nuclei show a moderate alkaline phosphatase reaction.

II. *Acid Phosphatase*

(*a*) *Oocyte development.* Quite contrary to alkaline phosphatase, acid phosphatase has a wide distribution in the *Drosophila* ovary. In ovarian follicles of all developmental stages, the oocyte, follicular epithelial cells and nurse cells are all very rich in acid phosphatase. The nuclear reaction is generally particularly strong and shows no variation throughout the development of the oocyte; on the other hand the cytoplasmic reaction is relatively weaker and varies between different developmental stages (especially in the nurse cells). Pl. 1, fig. 5, indicates the general features of acid phosphatase reaction of some young egg follicles.

There seems to be very little change of acid phosphatase activity in the follicular epithelial cells as the oocyte grows. Their nuclei stain as heavily as those of the nurse cells or oocytes, but their cytoplasmic activity is slightly weaker.

In the very young follicles, nurse cells are undergoing endomitosis and the resulting multiple chromatids show a strong tendency to remain together in definite groups (Painter and Reindorp, 1939). These polytene chromosomes give a very intense acid phosphatase reaction. Cytoplasmic activity of the nurse cells in these very young follicles is comparatively low. It increases substantially in the older follicles (stages 4 and 5), especially around the intracellular vacuoles and cell border. The activity falls off during stage 6, but the pycnotic nuclei remain very reactive.

As is evident from Pl. 1, fig. 5, the oocyte is very rich in acid phosphatase, both in the nucleus and cytoplasm. In the cytoplasm, the concentration of enzyme near the peripheral part of the egg is a distinct feature in the follicles of stages 4–6. The yolk granules as they are formed in this region are likewise endowed with high acid phosphatase activity. A similar concentration of enzyme is also noticeable around the vacuoles present in the ooplasm in stage 6. In mature oocytes cortical localization of acid phosphatase becomes less evident.

The distribution of acid phosphatase in the *Drosophila* ovary has also been studied by incubating fresh ovaries directly in lead glycerophosphate reagent. After half an hour of incubation, the nuclear reaction is already evident in the peripheral follicles of the ovaries, chiefly in the follicular epithelial cells and nurse cells. The slower reaction of the oocyte is perhaps due to the limitation set by the rate of penetration of the reagents. The reaction becomes more intense and definite as the duration of incubation increases. Figs. 6 and 7 on

Pl. 2 present the differences found between a group of experimental an control follicles after 2-hour incubation (sectioned materials after the sam period of incubation show practically no measurable activity). If one compar Pl. 2, fig. 6, with Pl. 1, fig. 5, it is evident that the differential concentration acid phosphatase between nucleus and cytoplasm as well as that between t cytoplasm of the nurse cells of different developmental stages are precise the same in both cases.

Acid phosphatase is also demonstrable both in the nucleus and cytoplas of the oviduct epithelium.

Like the ovary, the adult testis is rich in acid phosphatase. Spermatogon were found to contain less acid phosphatase than spermatocytes which, turn, are less reactive than spermatids and sperm. As regards mature sperm both the heads and the whole matrix in which the sperm are embedded see to contain the enzyme.

From this outlined description it is clear that the cytoplasmic localizatio of acid phosphatase during *Drosophila* oogenesis shows, on the whole, a clos correlation with the distribution of ribonucleic acid (Yao, 1950). Such correlation, however, does not exist in the case of spermatogenesis.

(*b*) *Embryonic development*. Acid phosphatase can be demonstrated in th developing embryos from cleavage up to the hatching of the larva. N apparent change in enzyme activity seems to occur during the whole embryon development, although irregular results have often been encountered due t the lability of this enzyme. Generally speaking, the most active site is th yolk which stains dark brown to black. Nuclei of embryo cells are the nex most active structures, giving a golden-brown appearance. Cytoplasm is th site of weakest activity, showing a light yellow to yellowish-brown colou Alternate sections as controls are always colourless.

In cleavage stages, acid phosphatase is mostly concentrated in the sub cortical plasma and in the yolk; whereas the egg cortex appears transparen with a slight yellowish tint and is possibly free from the enzyme. In dividin nuclei, chromosomes and spindle area are found to be moderately active.

In the single blastoderm stage, acid phosphatase is localized in the nucle of the blastoderm cells and in the yolk (Pl. 2, fig. 8). In this same figure stronger reaction in the 'innere Blastema' which corresponds more or less t the subcortical plasma in the cleavage stage is clearly visible. Throughout th gastrulation stage, the situation remains the same: a stronger reaction is foun in the yolk and in the cell nuclei (Pl. 2, fig. 9). No difference has been found between the cells of the three germ layers, nor is there any differential inten sity of reaction in the different parts of an embryo at this or any other late stages.

Pl. 2, fig. 10, is taken from a 12½-hour embryo. As can be seen in the figure the nuclear reaction in the nervous system, proventriculus, cephalopharyngea apparatus and hind-gut is still comparable to that of the early embryonic cells The mid-gut with its enclosed yolk forms the most active centre of acid phosphatase activity. Comparing this distribution with the alkaline phos-

phatase reaction of an embryo of the same age, a most noticeable difference is that the yolk and nerve-fibres give a moderate to strong acid phosphatase reaction but no trace of (yolk) or very weak (nerve-fibres) alkaline phosphatase activity.

Further difference between the behaviour of the two kinds of phosphatase during *Drosophila* embryogenesis is found in embryos prior to hatching. No decline of acid phosphatase activity has been noticed (Pl. 2, fig. 11). Both nuclear and cytoplasmic reactions are still demonstrable in every tissue. In the case of gut epithelia (including proventriculus and gastric caeca) and salivary glands, it is even possible that there is some increase in cytoplasmic activity.

Due to the extreme impermeability of the vitelline membrane, it was not possible to check the distribution of acid phosphatase in *Drosophila* embryo by incubating fresh unfixed material.

The high acid phosphatase activity during *Drosophila* embryogenesis and a similar high concentration of ribonucleic acid in *Drosophila* embryo cells (Yao, 1950) naturally suggests a functional correlation between these substances. Particularly illustrative are organs such as gut epithelia and salivary glands which show a parallel increase in cytoplasmic basiphily and acid phosphatase activity in later embryonic period. However, it should be pointed out again that such a correlation is not an absolute one, since both the yolk and nerve-fibre region are enzymatically active, but are void of ribonucleic acid.

Discussion

Perhaps the most interesting and significant fact emerging from the present study is the demonstration of the presence of two different patterns of phosphatase activity in *Drosophila* embryogenesis: acid phosphatase shows no change in activity, at least histochemically, throughout embryonic life; whereas alkaline phosphatase arises at a certain stage, increases in activity and then recedes (in most tissues). Quite recently, some preliminary data concerning the phosphatase activity in the early development of *Arbacia* has become available (Mazia *et al.*, 1948). They found that in the unfertilized eggs acid phosphatase predominates and its activity remains constant during early development. On the other hand, alkaline phosphatase activity remains constant until gastrulation, but increases very sharply after this event. The general pattern of phosphatase activity in the early development of *Drosophila* and of *Arbacia* is evidently much the same. In the case of the chick embryogenesis, the situation is somewhat different because the concentration of alkaline phosphatase is much greater than that of acid phosphatase from the very beginning (Moog, 1944, 1946). Nevertheless, the trend of the change of acid phosphatase activity between 2- and 12-day-old chick embryos is far less marked than that of alkaline phosphatase in the same period (Moog, 1946). From the above facts, it seems quite safe to say that acid and alkaline phosphatase are two different enzymes and play quite different physiological roles, even though they may be present simultaneously within the same cell.

The particular richness of acid phosphatase in the *Drosophila* embryo can be traced back to the development of the germ cells. In fact, I have found that high acid phosphatase activity is a characteristic of *Drosophila* gonads throughout the whole life-cycle of the fly. The physiological function of acid phosphatase is difficult to understand at present. In view of its constant activity during embryonic development, the enzyme probably has a more general relation to cell function than its suggested specific relation to proliferation and chemo-differentiation (Moog, 1944).

Whether or not alkaline phosphatase is present in small amounts in *Drosophila* ovaries and early embryos is not known. Quantitative determination of phosphatase activity, now contemplated, could provide an answer. At any rate, the histochemical evidence indicates that the concentration of the enzyme, if present, would be very low.

The factors which determine the origin (or activation?) of alkaline phosphatase in the ventral ectoderm after the contraction of the germ band as well as the differential rates of spreading after its first appearance are not clear. The fact that the spreading of enzyme activity is a gradual process seems to suggest that the primary factor involved in such spreading is actually direct contact between the active and inactive cells (in fact it reminds one of the process of infective colour transformation of guinea-pig skin (Medawar, 1947)). If this is true, the observed extero-interior gradient of alkaline phosphatase activity can be partially accounted for. Thus, the closer contact between hypodermal cells themselves than between a hypodermal cell and, for example, a nerve-cell would explain a quicker spreading within the hypodermis than spreading from the hypodermis to interior parts of an embryo. In this connexion, it is worthy of mention that the salivary glands which are still directly connected with the ventro-lateral ectoderm (shortly after the germ-band contraction stage) show a strong alkaline phosphatase reaction earlier than do other internal organs.

That contact is not the sole factor is indicated by the presence of an antero-posterior gradient which is also observable within the hypodermis itself. Evidently, there must exist within the embryo some physiological differences between the anterior and posterior parts and such differences control, in turn, the differential rate of enzyme spreading along the antero-posterior axis. The presence in the *Drosophila* embryo of a physiological gradient of some kind is also shown by Geigy's (1931) irradiation experiments. He discovered that the sensitization and de-sensitization of ventral ectoderm towards ultra-violet light exhibit a quite distinct 'thoraco-abdominal' gradation.

For various reasons it has been suggested in the first paper of this series that the stage of the contraction of the germ band divides, more or less, the growth phase of embryonic development of *Drosophila* from the phase of histological differentiation. The appearance of alkaline phosphatase during or immediately after the contraction of the germ band and its disappearance before hatching thus indicate that this enzyme is principally concerned with histo-differentiation. It is likely that the contraction of the germ band

represents a very important morphogenetic event and further investigation might be expected to reveal many biochemical differences between embryos before and after this stage.

Since Seidal's classical work on *Platycnemis*, it is generally admitted in experimental embryology that the organized development of insects involves the operation of two centres of organization, namely the activation centre and the differentiation centre. The presence of these centres in *Drosophila* has hitherto not been demonstrated though the work of Geigy (1931) points to their existence. Whether or not the centre of origin of alkaline phosphatase activity in the ventral ectoderm near to the future thorax represents the actual differentiation centre in *Drosophila* cannot yet be fully ascertained; but that it is so appears very likely.

Acknowledgement. The present work was undertaken at the suggestion of Professor C. H. Waddington, F.R.S., to whom the writer is deeply indebted for his encouragement and criticisms throughout. Acknowledgement should also be made to Mr. H. G. Callan and Dr. R. A. Beatty for their helpfulness and criticisms of the manuscript. To Mr. G. R. Knight, I owe gratitude for his assistance in photography. My thanks are due to the British Council for a grant which made this study possible.

Summary

1. *Drosophila* ovary and testis are very rich in acid phosphatase, but contain no histochemical trace of alkaline phosphatase. Thus the mature oocyte shows a strong acid phosphatase reaction both in the nucleus and cytoplasm. Sperm heads are equally reactive.

2. Acid phosphatase is demonstrable in *Drosophila* embryos from early cleavage up to the hatched larva. No striking change in enzyme activity has been observed during this period.

3. Alkaline phosphatase is not detectable in the first half of embryonic life. It suddenly appears in the ventral ectoderm near the future thorax during or shortly after the contraction of the germ band. The enzyme activity then spreads to the other parts of the embryo following definite patterns, until finally the whole embryo becomes active. The possible mechanism of the spreading of enzyme activity is discussed.

4. Alkaline phosphatase disappears in most tissues before hatching, but is retained in the gut epithelia, salivary glands, and Malpighian tubes. The relationship of this enzyme to histo-differentiation is suggested.

5. The centre of origin of alkaline phosphatase activity is considered as the 'differentiation centre' of the *Drosophila* embryo.

6. The high cytoplasmic acid phosphatase activity of the oocyte and nurse cells and a similar activity of the yolk in the developing embryos indicate that the enzyme plays some role both in the synthesis and in the degradation of yolk.

REFERENCES

Bartelmez, G. W., and Bensley, S. H., 1947. Science, **106,** 639.
Danielli, J. F., 1946. J. exp. Biol., **22,** 110.
Geigy, R., 1931. Roux Archiv., **125,** 406.
Mazia, D., Blumenthal, G., and Benson, E., 1948. Biol. Bull., **95,** 250.
Medawar, P. B., 1947. Biol. Rev., **22,** 360.
Moog, F., 1944. Biol. Bull., **86,** 51.
—— 1946. J. cell. & comp. Physiol., **28,** 197.
Painter, T. S., and Reindorp, E., 1939. Chromosoma, **1,** 276.
Stafford, R. O., and Atkinson, W. B., 1948. Science, **107,** 279.
Yao, T., 1950. (In the press.)

EXPLANATION OF PLATES 1 AND 2

G, cerebral ganglion or brain
hg, hind-gut
mg, mid-gut
P, proventriculus
ov, oviduct
sl, salivary gland
Vg, ventral nerve cord

Plate 1

Fig. 1. Oblique sagittal section of a 9-hour embryo showing the appearance of alkaline phosphatase in ventral hypodermis. The arrow-head indicates approximately the centre of origin of enzyme activity. × 120.

Fig. 2. Sagittal section of a 10–10½-hour embryo showing the spreading of alkaline phosphatase activity towards the interior and posterior parts of the embryo. × 120.

Fig. 3. Longitudinal section of a 14-hour embryo showing the distribution of alkaline phosphatase activity. × 120.

Fig. 4. Sagittal section of an embryo just before hatching. Note the alkaline phosphatase activity of the salivary gland and gut. × 120.

Fig. 5. Part of a section of a 24-hour ovary. Note the intense acid phosphatase activity of the nuclei of the nurse cells and oocytes. × 95.

Plate 2

For list of abbreviations, see Plate 1.

Fig. 6. Acid phosphatase reaction in fresh ovarian follicles incubated for 2 hours in lead glycerophosphate mixture. Compare with Pl. 1, fig. 5. × 82.

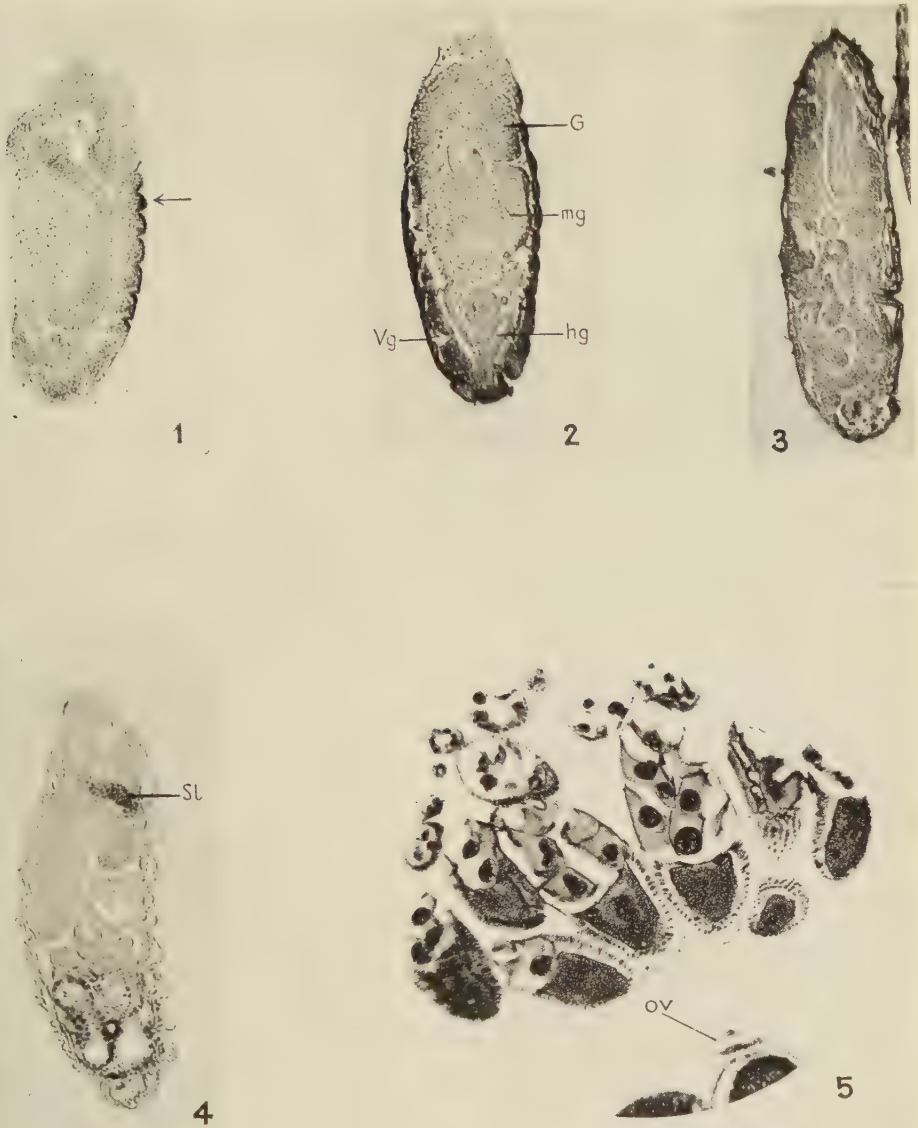
Fig. 7. Same as fig. 6, incubated in the presence of 0·01 M. fluoride. × 82.

Fig. 8. Longitudinal section of an embryo in the single blastoderm stage showing acid phosphatase distribution. × 120.

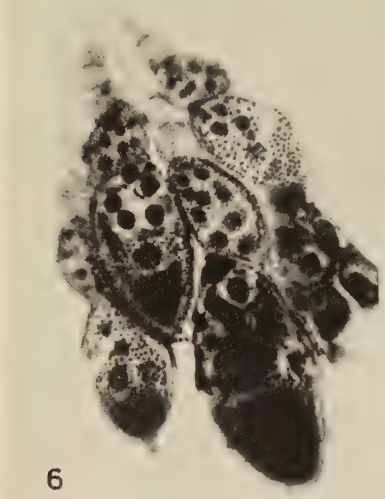
Fig. 9. Sagittal section of a 4½-hour embryo in an advanced stage of gastrulation showing acid phosphatase distribution. × 120.

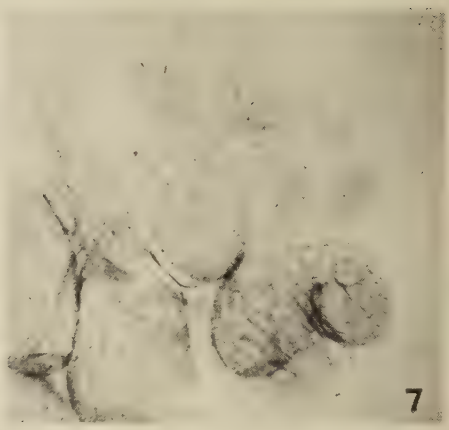
Fig. 10. Sagittal section of a 12½-hour embryo showing acid phosphatase distribution. × 120.

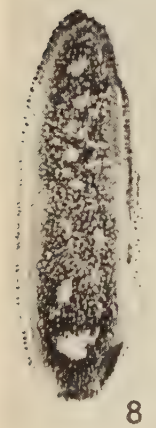
Fig. 11. Sagittal section of a 17½-hour embryo showing acid phosphatase distribution. The arrow-head indicates the point of puncture during fixation. × 120.

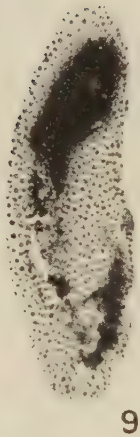


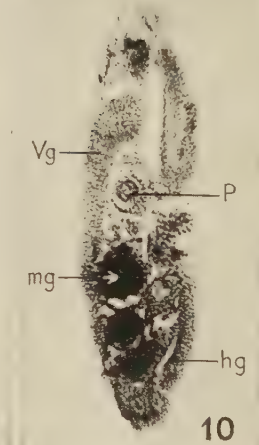
YAO: Alkaline and Acid Phosphatase—PLATE I











YAO: Alkaline and Acid Phosphatase—PLATE II

The Localization of Alkaline Phosphatase during the Post-embryonic Development of *Drosophila melanogaster*

BY

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With four Plates

IN the previous communication (Yao, 1949*b*), alkaline phosphatase has been shown to be in some way connected with histo-differentiation during *Drosophila* embryogenesis. The post-embryonic development of *Drosophila* includes, besides a period of larval growth, the phenomenon of metamorphosis which involves the destruction of most larval organs and the simultaneous development of imaginal organs from groups of embryonic cells known as imaginal disks. It is evident from Geigy's (1931) classical experiment that the *Drosophila* egg, at the time of fertilization, is already endowed with both the larval and the imaginal developmental patterns. From the standpoint of developmental physiology it would appear, therefore, that embryogenesis aims at the realization of the larval pattern, and post-embryonic development at that of the imaginal pattern. Since the basic processes through which these two developmental patterns are brought into reality are fundamentally the same, one might expect an active participation of alkaline phosphatase in metamorphosis as well as in embryogenesis. It is the purpose of the present paper to give an account of the activity of this enzyme in the larval and pupal life of the fly.

Material and Methods

Wild-type Oregon S stock of *Drosophila melanogaster* Meig. was used. In order to collect larvae of known age, 30–50 rapidly laying females were allowed to lay eggs in a fresh-food bottle for 1 hour. Larval age was computed from the time of egg laying, the total duration of embryonic life being 18–19 hours at $25 \pm 0{\cdot}2^{\circ}$ C. The limit of age-difference within such a group of larvae is approximately ± 1 hour. To obtain the requisite stages of prepupae and pupae, mature larvae prior to puparium formation were transferred to agar plates in Petri dishes. Following Robertson (1936), the moment when the anterior spiracles ceased to move was carefully recorded for a group of individuals and taken as zero hour.

Owing to the impermeability of the cuticle, the material requires to be pricked in order that the fixative may penetrate. In the cases of prepupae older than 4 hours, and pupae, the puparium case was carefully removed after fixation.

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Larvae, prepupae, and pupae of different developmental stages were often mixed before paraffin-embedding and then sectioned together so as to render the results comparable.

The technique used for the histochemical demonstration of alkaline phosphatase has been described in the previous paper (Yao, 1949*b*). However, the duration of incubation was extended to 12 hours in order to get the maximum possible reaction in tissues of low phosphatase content.

In the post-embryonic development of *Drosophila*, alkaline phosphatase activity shows a considerable degree of variation between different organs and between different morphological stages. In order to express such differences as objectively as possible, enzyme activity has been classified into three grades: these are by no means satisfactory but they are sufficient to describe some important changes of enzyme activity. A 'strong' reaction refers to a tissue or a part of a tissue which appears so black that its internal structure is often difficult to identify. A 'moderate' reaction describes material showing dark-brown precipitates in the nucleus and diffuse brownish to greyish colours in the cytoplasm. Histological details can easily be made out in these. A tissue is referred to as 'weakly' reactive when the nuclei only contain brownish precipitates.

Results

I. *Larval Development*

The localization of alkaline phosphatase has been studied in a series of *Drosophila* larvae from hatching up to puparium formation with an age interval of about 8 hours. The following general results are noteworthy: (i) during most of the 1st instar larval period, enzyme activity is very weak and is similar to that of a larva before hatching; it increases in the later part of 1st instar life (between the 16th and 24th hour after hatching): (ii) in 2nd instar larvae (approximately between the 24th and 48th hour), alkaline phosphatase activity is more readily demonstrable, and (iii) the activity in early 3rd instar larvae (from the 48th to 64th hour) is still not very different from that of 2nd instar larvae, but larvae which had ceased to feed and which were crawling about on the sides of the culture bottle at the time of fixation (generally between 72nd and 80th hour) give quite a different picture of enzyme distribution.

In general, nuclear alkaline phosphatase is demonstrable in every tissue, but cytoplasmic phosphatase shows a strict organ specificity. The differential high enzyme activity in the gut, salivary glands, and Malpighian tubes, as has been found in the larva before hatching, is maintained during the larval life.

The following account of enzyme distribution is based on the observations made on larvae aged between 16 and 64 hours.

Nervous System. Nerve-cells are moderately reactive. Mitoses were found during the larval period, especially among the giant ganglion cells. The phosphatase reaction of the nerve-fibres is weak.

Digestive System. The phosphatase reaction of the pharynx, proventriculus, gastric caeca, and hind-gut is weak: so also is the reaction of the cephalo-pharyngeal apparatus. In the mid-gut, the nuclei are moderately reactive and the striated border strongly so (Pl. 1, fig. 1, *mg*). In the cytoplasm of mid-gut cells, the enzyme is more concentrated on the side of the cell between the nucleus and striated border, as though it were being secreted into the intestinal lumen. Salivary glands are the most active organs (Pl. 1, fig. 2, *sl*). The enzyme is present in both the nucleus and cytoplasm as well as in the contents of the lumen. The activity is often so high after 12 hours of incubation that neighbouring tissues are affected by contamination. The presence of alkaline phosphatase in salivary glands has been previously reported (Danielli and Catcheside, 1945; Krugelis, 1945, 1946).

Malpighian Tubes. Moderate activity is found in the nucleus and some cytoplasmic granules. The brush border and sometimes the whole lumen are strongly reactive (Pl. 1, fig. 1, Pl. 2, fig. 7, *Ma*). The lumina of the anterior branches of the Malpighian tubes are much wider than those of the corresponding posterior branches, and their width increases as the larva grows. The contents in such dilated lumina stain in both experimental and control sections. Evidently, this is due to the presence of preformed calcium salts (Eastham, 1925).

Hypodermis. The phosphatase reaction varies from weak to moderate (Pl. 1, figs. 2, 3, *h*). It is possible that those hypodermal cells which are engaged in cuticle secretion show more phosphatase activity than those which are not so engaged. Oenocytes lying beneath the hypodermis give a moderate nuclear reaction only.

Tracheae. The epithelium as well as the cuticular intima are strongly reactive at the time of moulting (Pl. 1, fig. 3, *tr*). The strong positive reaction of the shed cuticle of the previous instar (*ti*) is clearly shown in the figure. Otherwise, the phosphatase reaction of the tracheae is moderate.

Muscles. In a fully developed muscle-fibre, nuclei and myofibrils show a moderate reaction, whereas the sarcoplasm is almost negative. The anisotropic disks of the myofibrils are far more reactive than the isotropic disks, giving thus a typical banded structure (Pl. I, fig. 4).

Fat-bodies. Fat-cells give a weak to moderate nuclear reaction, but their cytoplasm is negative (Pl. 1, figs. 2, 3; Pl. 3, fig. 13*a*). The stronger reaction of those fat-cells situated near to a very active organ (in Pl. 1, fig. 2) is due to contamination.

Gonads. Both ovaries and testes are weakly positive throughout larval life.

Imaginal disks. All imaginal disks give a moderate alkaline phosphatase reaction which is constant throughout the first and second larval instars and the early phase of the third instar. In general, the phosphatase reaction of imaginal disks is comparable to that of the nerve-cells and is stronger than that of the hypodermis from which they are mostly derived. This higher activity is evidently linked to the proliferative growth of the disks. Some

of these disks are shown in Pl. 1, fig. 2*a* and *b* (*Fr*, *W*). It should be pointed out that the difference in reaction intensity between the salivary glands and imaginal disks, while distinct in actual preparations, is not evident from the photographs. The alkaline phosphatase reaction of imaginal disks declines almost to zero in larvae preparing for puparium formation (Pl. 2, fig. 5).

Ring gland. From the embryological study of Poulson (1945), it is known that the ring gland of *Drosophila* constitutes a fusion between paired corpora cardiaca and a single corpus allatum. The alkaline phosphatase reaction of this organ is very weak in 1st instar larvae. It becomes moderately active in 2nd instar larvae (Pl. 1, fig. 2*b*, *Rg*), the enzyme activity being mostly confined to the nucleus: nuclei of the corpora cardiaca are much more active than the smaller nuclei of the corpus allatum. Parallel to the phenomenon observed in the case of imaginal disks, the enzyme activity of the ring gland is again very weak in the 3rd instar larvae (60, 72, and 80 hours old).

Heart and related structures. A moderate reaction is visible in the nuclei of the heart cells. Their cross-striated contractile fibrils react only weakly. Paired lymph-glands are moderately positive.

There are two groups of pericardial cells in the larva of *Drosophila*. About 16 pairs of large pericardial cells are situated on either side of the heart. The small pericardial cells, about 32 in number, are binucleate and are located between the brain and proventriculus. Although these latter bear no direct relationship to the heart, their phosphatase activity, intracellular ribonucleic acid distribution, and their behaviour during metamorphosis point to their similarity to the large pericardial cells. Both types of pericardial cells give a moderate alkaline phosphatase reaction during the larval period: a concentration of the enzyme around the cell membrane can often be noticed (Pl. 1, fig. 2*c*, *Sp*, Pl. 4, fig. 15, *Lp*).

It was stated at the beginning of this section that the distribution of alkaline phosphatase in late 3rd instar larvae (72–80 hours after hatching at $25 \pm 0{\cdot}2^{\circ}$ C.) differs from that in young larvae. The most significant differences are the general decrease of alkaline phosphatase activity in the internal organs and the simultaneous increase in the hypodermis (Pl. 2, fig. 5, *Pm+h*) and possibly also in the muscles below. For example, nervous system, imaginal disks, and ring gland which show moderate activity in the 2nd and early 3rd instar life are now only weakly reactive in their nuclei. Some decrease of enzyme activity probably also occurs in the salivary glands, mid-gut, Malpighian tubes, and pericardial cells, although they are still the most active organs at this stage. The cause of this change of alkaline phosphatase activity is not known but, since the larvae are preparing for pupation, it is natural to connect the high alkaline phosphatase activity of the hypodermis with the formation of the puparium. How close such a connexion may be can only be answered by transplantation of the ring gland.

II. *Prepupal and Pupal Development (Metamorphosis)*

1. *General Consideration*

Since the processes of metamorphosis which involve histolysis and histogenesis start in mid-prepupal life, it seems to be more appropriate to treat together the results obtained from the cytochemical studies made on the prepupae and pupae.

Within the first few hours after the anterior spiracles cease to move, the alkaline phosphatase reaction of a prepupa is rather weak, just like that found in a larva prior to puparium formation (Pl. 2, fig. 6, compare with fig. 5). Salivary glands, hypodermis, muscles, mid-gut, Malpighian tubes, pericardial cells, and lymph-glands are among the relatively active organs. The reaction of the nervous system, ring gland, and all imaginal disks is still exceedingly weak. However, from the 5th hour onwards, there begins a definite increase in reaction intensity. This is especially noticeable in the organs having weak enzyme activity such as the nerve-cells, ring gland, and imaginal tissues.

In prepupae prior to head eversion (Pl. 2, fig. 7), the relative distribution of alkaline phosphatase activity is as follows: salivary glands, yellow body, larval hypodermis, anterior portions of the tracheae, pericardial cells, lymph-glands, and histolysing structures (detached head and thoracic muscles and detached hypodermal cells) all show a moderate to strong reaction; fat-bodies, abdominal muscles, hind-gut, posterior parts of the larval tracheae, and gonads react very weakly. Because of this differential activity, the anterior part of the prepupa is decidedly more reactive than the posterior part. This also is clearly indicated in the same figure.

When the larval and imaginal components of an organ are lying side by side as, for example, in the case of the hypodermis, mid-gut, or salivary glands, it is generally true that the larval cells are much richer in alkaline phosphatase content than are the imaginal cells (see Pl. 3, fig. 12).

If the increased alkaline phosphatase activity due to histolysis in the late prepupae is not taken into account, the enzyme activity of the prepupa is similar to that of the larva rather than to that of the pupa.

Pupation occurs between the 11th and 12th hour (at $25 \pm 0{\cdot}2^{\circ}$ C.) after the larva has become quiescent. The visible morphological changes which mark its beginning are the sudden eversion of the whole cephalic complex, the breakdown of a part of the fat-bodies into individual cells, and the pouring of the latter into the newly formed head. A comparative study of the alkaline phosphatase reaction of prepupae just before head eversion and that of pupae immediately after head eversion has revealed a definite and sudden increase of enzyme activity accompanying pupation. Since a moderate nuclear reaction is present in every tissue before pupation, the most noticeable increase is found in the cytoplasm.

Just after head eversion, the phosphatase reaction in the head and thorax is very strong (Pl. 2, fig. 8), but comparatively weak in the abdomen except

in those parts adjoining the thorax. A few hours later, the reaction in the abdomen increases, due to advancing histolysis of the abdominal muscles, posterior larval tracheae, and hypodermis, as well as fat-bodies. This high phosphatase activity is maintained for the first 24 hours or so after head eversion (Pl. 3, fig. 10), during which period histogenesis and histolysis are progressing rapidly. Subsequently, the enzyme activity declines, starting in the head and thorax. In pupae older than 48 hours and in newly emerged flies, the phosphatase reaction is very weak in the head and thorax, nerve-cells being an exception. The reaction in the abdomen is, however, still moderate to strong (Pl. 3, fig. 11). This relatively high enzyme activity in the abdomen of a late pupa is probably connected with: (i) the late histogenesis of the abdominal muscles; (ii) the descending of phosphatase-active mid-gut and yellow body into the abdomen; (iii) the delayed decline of enzyme activity of the gonads, and possibly (iv) the slow histolysis of the abdominal fat-bodies.

2. *Alkaline Phosphatase Reaction of Different Organ Systems*

The following is an account of the change of alkaline phosphatase activity in different organ systems during metamorphosis:

(*a*) *Nervous system.* The phosphatase reaction is very weak in the early, and moderate in the late prepupal periods. It is greatly enhanced after pupation (compare Pl. 2, figs. 7, 8, and 9*a*, and Pl. 3, fig. 9*b*). The nerve-fibre region now also becomes moderately reactive. This moderate activity remains unchanged until about the 48th hour after puparium formation. The reaction is again very weak in pupae aged between 52 and 68 hours. After the 72nd hour, a secondary increase of alkaline phosphatase activity occurs in the nervous system. The nerve-cells of the brain proper and ventral ganglion give a moderate reaction; the middle and inner optic ganglia a weak to moderate one; the middle and the outer optic ganglia a very weak, or even negative one. This condition persists in pupae prior to emergence and in 24-hour-old adult flies.

(*b*) *Antennae and compound eye.* Both antennal and eye disks are almost inactive in young prepupae (Pl. 2, fig. 6, *Fr*); but they are moderately active in 5- to 11½-hour prepupae. After pupation, the phosphatase reaction is intensified just as is that of the rest of the head ectoderm (Pl. 2, fig. 8, *Od*). At this time, antennal disks are in the form of two thickened ectodermal plates. In a few hours, the two major antennal joints appear by processes of folding and extension. Antennae are well defined in a 26-hour pupa. During this period, alkaline phosphatase reaction is moderate to strong. It declines afterwards and becomes almost completely absent in 44-hour pupa. Johnston's organs are recognizable in 72-hour pupa, but they show only a weak nuclear reaction.

Similarly, eye disks are also represented by two thickened ectodermal plates, each consisting of several rows of cells at the time of the head eversion. The development of the ommatidia in the eye of *Drosophila melanogaster* has

been carefully studied by Pilkington (1941). From his account it is obvious that the formation and the histogenesis of the individual components of an ommatidium—retinulae, cone cells, pigment cells, and corneal lenses—take place largely in the first 2 days after head eversion (i.e. between 12- and 60-hour pupae). Moderate to strong phosphatase reaction of the eye rudiments is a constant feature in pupae aged between 12 and 42 hours. The enzyme activity then falls and only a weak nuclear reaction is demonstrable in 60-hour and older pupae and in freshly emerged flies.

(*c*) *Wings and legs.* These organ disks show very weak phosphatase reaction in the early prepupal period (Pl. 2, fig. 6, *W*, *L*). They become moderately reactive after their eversion in the mid-prepupal stage. Accompanying pupation there is an increase of both nuclear and cytoplasmic phosphatase in these organs (compare Pl. 2, figs. 7, 8, 9*a*, and Pl. 3, fig. 9*b*). This high phosphatase activity prevails in both organs up to about 36-hour pupae (Pl. 3, fig. 10, *L*). Inactivation or rather destruction of the enzyme then begins, leading first to a moderate (48-hour pupa) and finally to a weak reaction (72-hour pupa). Waddington (1940*a*) has made an extensive study of the development of normal and mutant wings of *Drosophila*. From his description, it is evident that the most important morphogenetic events in normal wing development occur during the 'definitive wing stage' (stage P2 (18- to 45-hour) in his paper). The observed strong alkaline phosphatase activity of the wings in 12- to 36-hour pupae and a moderate activity in 36- to 48-hour pupae therefore strongly suggest that the enzyme is particularly concerned with histo-differentiation.

During the transition period (36- to 72-hour), it has been noticed that the distal parts of these organs often lose their enzyme activity earlier than the corresponding proximal parts; and that the wings lose their enzyme activity earlier than the legs.

Residual nuclear phosphatase activity is still present in the wings and legs of newly emerged flies. Trichogenic cells are very difficult to recognize in slides prepared to demonstrate alkaline phosphatase.

The development of leg muscles follows the same temporal course as the development of most thoracic muscles and will be discussed in the section on muscles.

(*d*) *Hypodermis.* Following the strong phosphatase reaction before puparium formation, the hypodermis is always an active site of phosphatase activity in prepupae (Pl. 2, figs. 6, *pm*–*h*; 7, *h*). During the 12 hours of prepupal life, all larval thoracic hypodermis is replaced by an imaginal one, whereas in the abdomen the hypodermis remains mostly larval, with only a few scattered groups of imaginal cells. The phosphatase reaction is very strong in the larval cells, but only moderate in the imaginal cells.

Soon after pupation, the phosphatase reaction of the head and thoracic hypodermis becomes very strong and remains so until about the 38th hour (Pl. 2, fig. 8, Pl. 3, figs. 9*b*, 10). The reaction gradually fades away and becomes absent in 60-hour pupae.

On the other hand, no change in enzyme activity has been observed in the abdominal hypodermis shortly after pupation. The contrast between the activity of the larval and imaginal cells can still be seen (Pl. 3, fig. 12). Six hours later, this differential reaction becomes less and less striking due to increasing enzyme activity in the imaginal cells. When the abdominal hypodermis is completely replaced, its phosphatase reaction is very strong (Pl. 3, fig. 10). Folding of the hypodermis to form tergites and sternites occurs in 36-hour to 48-hour pupae. The phosphatase reaction is strong during this period. It becomes moderate after the 48th hour and weak after the 60th hour.

Trichogenic cells or the histoblasts of the hairs and bristles in the head and thorax can be readily distinguished from the rest of the hypodermal cells by their larger size and stronger nuclear phosphatase reaction. In the abdomen they are smaller and hence are hard to discriminate from the ordinary hypodermal cells.

(*e*) *Muscles*. Larval muscles are completely destroyed during metamorphosis. They give a weak to moderate phosphatase reaction in early prepupae. As soon as they are detached and undergo histolysis, the sarcolytes show a moderate to strong reaction. The abdominal muscles, however, undergo histolysis a few hours after pupation: thus strongly reactive sarcolytes can be found in the abdomen of 15- to 24-hour pupae. A similar increase of alkaline phosphatase activity occurs in the muscular coats of the gut when these are undergoing histolysis.

Myocytes in the thorax of a late prepupa are moderately active (Pl. 2, fig. 7, *My*). After head eversion, intense proliferation goes on and the phosphatase reaction becomes very strong (Pl. 3, fig. 9*b*, *My*). The resulting mass of myocytes is more or less in the form of a syncytium. In 20- to 24-hour pupae, the first sign of muscle differentiation—the formation of myofibrils and the arrangement of the nuclei into parallel rows—becomes visible. Further differentiation between 24- and 48-hour pupae involves the growth of the muscle-fibre as a whole and the continuous formation of myofibrils. As a rule, the phosphatase reaction of a developing muscle is very strong in 12- to 36-hour pupae but only moderate in 38- to 52-hour pupae. Furthermore, the development of the dorsal thoracic muscles proceeds several hours ahead of that of the other thoracic, head, and leg muscles. In 54- to 56-hour pupae, the dorsal thoracic muscles almost reach their final length and their phosphatase reaction is very weak.

Myofibrils give a positive phosphatase reaction. When the isotropic and anisotropic disks become differentiated, a slightly stronger reaction is found in the anisotropic disks. This cross-striation appears in the dorsal thoracic muscles in 48- to 52-hour pupae; in other muscles it develops somewhat later.

The development of the abdominal muscles occurs much later. Definite myocytes, each regularly spaced, can be readily seen in 48-hour pupae. The myofibrils first appear in 54- to 56-hour pupae. When the phosphatase activity of the thoracic, head, and leg muscles is already in decline, abdominal

muscles show a very strong reaction in 48- to 72-hour pupae. They are still moderately active in 80-hour pupae.

In newly emerged flies, residual phosphatase activity can still be demonstrated in the nuclei and myofibrils of all muscles.

(*f*) *Digestive system.* The phosphatase reaction of the fore-gut is rather weak in prepupal stages. Unlike the hypodermis, the larval and imaginal components show very little difference in enzyme activity. The fore-gut gives a strong reaction on the first day after pupation (Pl. 3, fig. 10, *Ph*). The reaction is weak to moderate on the second and third days of pupation. The crop, which is entirely an imaginal organ, is strongly reactive before the 38th hour and moderately so afterwards.

Because of the high phosphatase activity of the salivary glands in prepupae, it is not certain whether their enzyme activity is also enhanced after pupation. Nevertheless, fragments of the glands after their disintegration in 15-hour pupae show a very strong reaction (Pl. 3, fig. 9*b*, *sl*) until finally they disappear. The imaginal salivary glands are very difficult to trace in young pupae because of the strong phosphatase reaction of the thorax as a whole. They are found to be strongly positive in 28- to 48-hour pupae. Moderate reaction is retained in late pupae and in the adult flies.

The imaginal mid-gut formed in the early phase of prepupal life gives a weak alkaline phosphatase reaction in comparison with the strong reaction of the yellow body (Pl. 2, fig. 7, *Mg* and *y*). In the latter structure, the higher enzyme activity in the striated border of the larval gut cells is still recognizable. Pupation brings about a marked increase of phosphatase activity in both the imaginal mid-gut and yellow body (Pl. 2, fig. 8). Contrary to most other organs, the mid-gut does not show any appreciable decrease of enzyme activity in the second and third days after head eversion.

The moderate reaction of the larval hind-gut in prepupae changes into a stronger one in 16- to 32-hour pupae as histogenesis and histolysis set in. Moderate phosphatase activity is found in the hind-gut and rectal papillae at the time of emergence.

(*g*) *Malpighian tubes.* According to Robertson (1936), the larval organ transforms directly into the adult one without any visible morphological changes. In correspondence with this, it was found that the phosphatase reaction of the Malpighian tubes of prepupae and pupae is the same as that found in the larvae. The nuclear reaction is moderate, whereas the brush border and many cytoplasmic granules react very strongly. However, variations have also been found as, for example, segments of the tubes showing no reaction in the brush border, or segments free from active cytoplasmic granules.

The contents of the dilated anterior branches show positive reaction in both experimental and control sections.

(*h*) *Fat-bodies.* Since fat is the main energy source required for the morphogenetic processes during metamorphosis, the fat-bodies are mostly broken down after pupation. In the larval and prepupal periods, fat-cells are arranged into definite sheets, and their phosphatase reaction is moderate in the nuclei

but almost nil in the cytoplasm (Pl. 3, fig. 13*a*). Accompanying head eversion, there is a sudden dissolution of the fat-bodies and a concomitant increase of alkaline phosphatase activity in isolated fat-cells (Pl. 4, fig. 13*b*). The increase is mostly confined to the cytoplasm, where the enzyme is localized in the protoplasmic mesh-work separating the numerous globules. These individual fat-cells soon undergo histolysis and dissolve away.

On the whole, the histolysis of the fat bodies is a gradual process, for the thoracic fat-bodies are always first attacked and the most posterior abdominal fat-bodies last. Thus, the abdominal fat-bodies of a pupa shortly after pupation maintain their layered structure and show no increase of phosphatase activity. A similar gradient in the histolysis of the fat-bodies has been observed during the metamorphosis of *Calliphora* (Perez, 1910).

At the end of the first day after head eversion, the layered structure of the fat-bodies in the abdomen has mainly vanished. However, not all larval fat-cells are destroyed during the pupal stage. Those partially changed fat-cells left behind in the abdomen still give a moderate phosphatase reaction at the time of emergence. Further histolysis of these cells takes place during adult life.

The imaginal fat-bodies can readily be recognized in 60-hour pupae. They appear as groups of small cells situated beneath the hypodermis and give a moderate phosphatase reaction in the nuclei.

(*i*) *Tracheae.* The imaginal buds of the tracheae can be traced to the early second instar larvae. They are located near the anterior spiracles and show a moderate nuclear phosphatase reaction. During the prepupal stages, proliferation of the imaginal tracheal cells and histolysis of the larval tracheae are the main features. In 5-hour or older prepupae, the anterior portions of the tracheae give a very strong phosphatase reaction (Pl. 2, figs. 7, 9*a*, *tr*, *Tr*), but the posterior trunks (approximately one-third of the total length) invariably show a very weak reaction.

Just after pupation the prothoracic spiracles, main trunks in the thorax, and the principal branches to the head are all composed of imaginal cells. The anterior commissure and the main trunks in the anterior part of the abdomen are still larval. Both the imaginal and larval tracheae are strongly reactive (Pl. 2, fig. 8, *Tr*). The posterior larval trunks in the abdomen first become solid cords and then undergo histolysis in 14- to 24-hour pupae, with an accompanying rise in phosphatase activity.

The lateral spiracles as seen in the prepupae give a weak to moderate nuclear reaction. They become strongly positive after pupation.

The phosphatase activity of the tracheae and tracheoles, very strong the first day after head eversion, decreases in the second day. In 72-hour or older pupae the tracheae of the head and thorax are only weakly reactive, whereas those in the abdomen, in keeping with the activity of other abdominal organs, are moderately active.

(*j*) *Ring gland.* As in the imaginal disks and nervous system, the phosphatase reaction of the ring gland is almost absent in 0- to 5-hour prepupae.

After the 5th hour, enzyme activity increases. The corpora cardiaca now give a moderate reaction, though the corpus allatum is only weakly positive (Pl. 4, fig. 14). This is exactly the same situation as was found in second instar larvae.

Within the first few hours after head eversion there is no change of phosphatase activity of the ring gland. However, the whole gland becomes very reactive in 18- to 38-hour pupae, no longer showing a difference in activity between the corpora cardiaca and corpus allatum. The ring glands of 46-, 54-, 62-, 66-, and 72-hour pupae show moderate activity. At the time of emergence, the phosphatase reaction is still demonstrable in the middle of the gland.

In 80-hour or older pupae, the cells of the larval corpora cardiaca show a reduction in nuclear size and cytoplasmic content. In some cases the beginning of nuclear pycnosis has been observed. These are probably the preludes to their actual histolysis which takes place after emergence (Vogt, 1941, 1942*a*).

(*k*) *Heart and related structures.* The larval heart transforms directly into that of the adult with a change of form (Robertson, 1936). The phosphatase reaction of the heart in prepupal stages is the same as that of the larvae. The enzyme activity, as in other organs, increases after pupation. Both the nuclei of the cardiac cells and the muscle-fibres are active (Pl. 4, fig. 15, *Dv*).

The lymph-glands or the 'blood-forming organ' of Stark and Marshall (1930) give a moderate phosphatase reaction in the prepupal period. In pupae shortly after head eversion, two pairs (one pair according to Robertson) of such glands are found (Pl. 4, fig. 15, *lg*). They do not show any increase of phosphatase activity and disappear before the 18th hour.

The two groups of pericardial cells persist throughout metamorphosis and are present in adult flies. During the prepupal period their phosphatase reaction is moderate. Accompanying pupation, the increase of cytoplasmic phosphatase in these pericardial cells is very marked (Pl. 4, fig. 15, *Lp*). In the case of the large pericardial cells, this increase is especially clear in 18- to 32-hour pupae, for they appear as solid black bodies (Pl. 3, fig. 10, *Lp*). Both the large and small pericardial cells show a moderate phosphatase activity in the mid-pupal period. In later pupal stages the phosphatase reaction becomes rather weak and remains so in the emerged flies.

(*l*) *Gonads.* During metamorphosis, the ovary and testis show similar behaviour in phosphatase activity. Like those in the larvae, they react very weakly in the prepupal stages and exhibit a delayed response to the general increase of phosphatase activity after pupation. Together with the abdominal fat-bodies they thus constitute those structures which give a relatively weaker reaction in the early pupae. A very definite increase of phosphatase activity is, however, noticeable in the 32-hour pupae, when the gonads are found to be already joined by the genital ducts. From this stage onward the ovary and testis always give a moderate to strong phosphatase reaction until the time of emergence.

Pl. 4, fig. 16, is taken from a 32-hour male pupa showing the enzyme distribution in the testis and vas efferens. It is evident from the figure

that alkaline phosphatase is more concentrated in the nucleus than in the cytoplasm of the spermatocytes. The sperm heads are also strongly reactive.

In the ovary, the enzyme is present in the ovarian cords and the surrounding somatic tissues in the early developmental stage (Pl. 4, fig. 17). When the egg follicles begin to differentiate after the 72nd hour, phosphatase is also found to be mostly localized in the nuclei of the follicular cells, nurse cells, and oocytes (Pl. 4, fig. 18).

In the light of the above evidence, the negative alkaline phosphatase reaction of adult *Drosophila* gonads (Yao, 1949*b*) indicates that the enzyme is either destroyed or inactivated soon after emergence.

The development of the external genitalia and other accessory organs has not been followed in detail. The phosphatase reaction of the abdominal disks which give rise to these structures is very weak in the prepupal period (Pl. 2, fig. 7, *ad*). It increases at about 6 hours after head eversion, parallel to the general increase of enzyme activity in the imaginal hypodermis of the abdomen. In 32-hour pupae the well-formed genital ducts are as reactive as the gonads (Pl. 4, fig. 16, *Ve*).

Discussion

(1)

The interesting correlation between phosphatase activity and growth and differentiation was first demonstrated in chick embryogenesis by Moog (1944). She has since discussed the phenomenon at considerable length in her recent review (Moog, 1946). Subsequently, similar evidence has been briefly presented in the early development of amphibia (Brachet, 1946) and sea-urchin (Mazia *et al.*, 1948). The present observation on the post-embryonic development of *Drosophila*, together with the previous data on embryogenesis (Yao, 1949*b*), supports and strengthens this general correlation. Judging from the wide diversity of the taxonomic level of the animals so far studied, it seems very likely that high phosphatase activity in embryonic development (or the like, as in insect metamorphosis) is a universal phenomenon.

During embryogenesis, alkaline phosphatase appears immediately after the contraction of the germ band and disappears in most tissues before the hatching of the larva. This fact tends to indicate that the enzyme is primarily concerned with histo-differentiation (Yao, 1949*b*). In post-embryonic development, the alkaline phosphatase activity of the imaginal disks illustrates the same principle. In the larval and prepupal periods, their moderate enzyme activity is obviously linked to their proliferative growth. Then the activity suddenly increases at the time of pupation and maintains itself at this high level for the next day and a half. After that, alkaline phosphatase decreases in most organs, but remains at a high level in certain others. Considering now the developmental status of the imaginal organs at the time of pupation, the results of transplantation-work on the organ disks (Bodenstein, 1943; Vogt, 1943) certainly indicate that they have already passed their stage of

invisible or chemo-differentiation. Consequently, the increased alkaline phosphatase activity must also be concerned with histo-differentiation. In this connexion, the observed fact that the increased fraction is mainly cytoplasmic phosphatase is important, since differentiation is primarily a cytoplasmic process.

The similar behaviour of alkaline phosphatase during embryogenesis and metamorphosis not only suggests the basic identity between the processes underlying the realization of the larval and imaginal developmental patterns, but also lends support to the general belief that the larval period is a period of growth and that of metamorphosis a period of differentiation (see Bodenstein, 1942).

Furthermore, the general resemblance of alkaline phosphatase activity of the prepupa to that of the larva supports the conclusion derived from purely morphological evidence that the prepupa is actually an intrapuparial larval instar (Robertson, 1936).

(2)

The relation between phosphatase activity and cellular degeneration has so far not been fully recognized. The nearest example in the existing literature (to my knowledge) is perhaps the facts discovered by Bodian and Mellors (1944) in nerve degeneration. Even here, the increased acid phosphatase activity is attributed to the resynthesis of Nissl bodies rather than to their destruction (Bodian, 1947). In the present study it was found that the increase of alkaline phosphatase activity is almost concomitant with the actual progress of histolysis. This happens in every larval organ that is going to be destroyed, but it is especially clear in the case of fat-bodies. Acid phosphatase is also very active in histolysing tissue fragments (unpublished data).

As histolysis of fat-cells sets in after pupation, the increase of cytoplasmic alkaline phosphatase is so sudden and so marked that one is inclined to think that the increased portion is due to the activation of pre-existing enzyme molecules. Whether this represents the general mechanism by which the increased activity after pupation can be accounted for, and whether there occurs at the same time some *de novo* synthesis of the enzyme or not, are questions very hard to answer at the present stage. In this connexion it would also be interesting to know if the sudden increase of alkaline phosphatase after pupation is a hormone-controlled process.

(3)

Another aspect of phosphatase activity which I would like to discuss is its normal physiological function. Thus, the concentration of alkaline phosphatase in the striated border of the mid-gut and in the brush border of the Malpighian tubes is of some comparative interest, since analogous localization in the mammalian intestinal epithelium and kidney tubules is well known. Whether or not this means that a similar transport function is played by this enzyme in *Drosophila* is difficult to say in view of our limited knowledge about

insect digestion and excretion. Nevertheless, the similar enzyme localization does indicate such a possibility.

One component of the insect cuticle is the nitrogenous polysaccharide, chitin. As a corollary to the current conception of biosynthesis (Lipmann, 1941), the synthesis and breakdown of chitin may well involve phosphorylation processes as in the case of glycogen. The strong alkaline phosphatase reaction of the tracheal epithelium and its cuticular intima is therefore what one would expect in considering the complementary function of phosphatase in the complex phosphorylation processes. Similarly, the high alkaline phosphatase activity of the hypodermis during the formation of the puparium in the larvae, and that of exoskeleton in the pupae, can be interpreted on the same basis.

The high alkaline phosphatase activity of the pericardial cells during prepupal and early pupal periods, and the persistence of these cells throughout metamorphosis, suggests that they are probably secretory organs. This contention is supported by the following additional facts (unpublished data): (*a*) they show definite cycles of change of cell size in the course of metamorphosis; (*b*) secretion vacuoles have been observed in them in the larvae prior to puparium formation, and in prepupae and pupae; (*c*) a distinct sexual dimorphism has been found in the large pericardial cells, both during metamorphosis and in the adult flies. In this connexion it is interesting to note that Bodenstein (1943) (in the course of his extensive study of the physiology of the *Drosophila* ring gland) came to the conclusion that ring-gland hormone does not act directly on transplanted organ disks but, rather, indirectly through the intervention of some factors in the host. Therefore, it occurs to me that the pericardial cells are the most likely organs which cause such an interaction complication. In fact, an interaction between different endocrine glands in Orthoptera has been reported (Pflugfelder, 1939). However, it is not possible at present to compare these pericardial cells in *Drosophila* with the pericardial gland in *Dixippus* (Pflugfelder, 1938). It can only be stated that the pericardial cells are similar to the pericardial gland in showing sexual dimorphism, but differ from the latter by their persistence in adult flies.

From the transplantation work of Hadorn (1937), Nyst (1941), Bodenstein (1943, 1944), and Vogt (1942*b*, 1943), it is certain that the ring gland in *Drosophila* produces both moulting and metamorphosis hormones. The individual roles played respectively by the corpora cardiaca and corpus allatum are, however, not clear. Relying upon morphological (1942*b*) and experimental (1943) evidence, Vogt is of the opinion that the corpora cardiaca ('grosse Ringdrussenzelle' in her paper) are more concerned with metamorphosis than is the corpus allatum. The data presented in this paper seem to support her conclusion, if the higher alkaline phosphatase activity and ribonucleic acid content (unpublished data) of the corpora cardiaca in the larval and prepupal periods can be considered as indices of a higher level of physiological function. On the other hand, the present data also indicate that the corpus allatum becomes more active 6 hours after head eversion, if the above assumption is correct.

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Summary

1. The localization of alkaline phosphatase during the post-embryonic development of *Drosophila melanogaster* has been described.

2. In the larvae, nuclear phosphatase is always demonstrable, but cytoplasmic phosphatase shows a more restricted distribution. Salivary glands, mid-gut, Malpighian tubes, and pericardial cells are richest in cytoplasmic phosphatase.

3. The larva prior to puparium formation is characterized by a decrease of alkaline phosphatase in the internal organs with a simultaneous increase in the hypodermis.

4. The phosphatase data support the view that the prepupa is actually an intrapuparial larval instar.

5. Pupation is accompanied by a very noticeable increase of alkaline phosphatase which is mainly confined to the cytoplasm. The high enzyme activity is maintained for the first day and a half after head eversion: there is a subsequent decline until at the time of emergence most organs are inactive. However, certain organs retain their alkaline phosphatase activity.

6. As in embryogenesis, alkaline phosphatase seems to be more concerned with histo-differentiation than with chemo-differentiation.

7. Alkaline phosphatase (and also acid phosphatase) actively participates in the process of histolysis or cellular degeneration.

8. The alkaline phosphatase activity of the pericardial cells, together with other morphological evidence, indicates that these cells are endocrine organs which play important roles in *Drosophila* metamorphosis.

9. Cytochemical evidence suggests that alkaline phosphatase in *Drosophila* is probably playing a part in the carriage of organic substances across the membrane barrier.

REFERENCES

Bodenstein, D., 1942. Symp. quant. Biol., **10,** 17.
—— 1943. Biol. Bull., **84,** 34.
—— 1944. Ibid., **86,** 113.
Bodian, D., 1947. Symp. Soc. exp. Biol., **1,** 163.
—— and Mellors, R. C., 1944. Proc. Soc. exp. Biol. Med., **55,** 243.
Brachet, J., 1946. Experientia, **2,** 143.
Danielli, J. F., and Catcheside, D. G., 1945. Nature, **156,** 294.
Eastham, L., 1925. Quart. J. micr. Sci., **69,** 385.
Geigy, R., 1931. Roux Archiv., **125,** 406.
Hadorn, E., 1937. Proc. Nat. Acad. Sci., **23,** 478.
Krugelis, E. J., 1945. Genetics, **30,** 12.
—— 1946. Biol. Bull., **90,** 220.

Lipmann, F., 1941. Adv. Enzymol., **1,** 99.
Mazia, D., Blumenthal, G., and Benson, E., 1948. Biol. Bull., **95,** 250.
Moog, F., 1944. Ibid., **86,** 51.
—— 1946. Biol. Rev., **21,** 41.
Nyst, R. H., 1941. Ann. Soc. R. Zool. Belg., **522,** 74.
Perez, C., 1910. Arch. de Zool. exp., **4,** 1.
Pflugfelder, O., 1938. Z. wiss. Zool., **151,** 149.
—— 1939. Ibid., **152,** 384.
Pilkington, R. W., 1941. Proc. Zool. Soc. Lond. A, **111,** 199.
Poulson, D. F., 1945. Trans. Conn. Acad. Arts Sci., **36,** 449.
Robertson, C. W., 1936. J. Morph., **59,** 207.
Stark, M. B., and Marshall, A. K., 1930. Jour. Amer. Inst. Homeop., **23,** 1204.
Vogt, M., 1941. Biol. Zbl., **61,** 148.
—— 1942 *a*. Roux Archiv., **141,** 424.
—— 1942 *b*. Ibid., **142,** 131.
—— 1943. Biol. Zbl., **63,** 395.
Waddington, C. H., 1940. J. Genet., **41,** 75.
Yao, T., 1949 *b*. (In the press.)

EXPLANATION OF PLATES

Abbreviations

ad, abdominal disk
An, antennal disk
c, cuticle
Ca, corpus allatum
Cc, corpora cardiaca
Dv, heart or dorsal vessel
f, larval fat-body
fc, histolysing fat-cell
Fr, frontal sac
G, cerebral ganglion or brain
h, larval hypodermis
H, imaginal hypodermis
Ha, imaginal bud of haltere
hg, larval hind-gut
L, section of leg
L1, imaginal disk of prothoracic leg
L2, imaginal disk of mesothoracic leg
L3, imaginal disk of metathoracic leg
lg, lymph-gland
Lp, large pericardial cell
lu, leucocyte or phagocyte
m, larval muscle
M, imaginal muscle
Ma, Malpighian tube
Mc, mesenchyme cell
mg, larval mid-gut
Mg, imaginal mid-gut
My, myocyte
Od, ovary
Oes, oesophagus
Ov, oviduct
P, proventriculus
Ph, pharynx
pm, puparium
Rg, ring gland
S, mature sperm
sc, sarcolyte
sl, larval salivary gland or its histolysing fragment
Sl, imaginal salivary gland
Sp, small pericardial cell
Te, testis
ti, chitinous intima of trachea
tr, larval trachea
Tr, imaginal trachea
Ve, vas efferens
Vg, ventral ganglion or ventral nerve cord
W, wing or its imaginal disk
y, yellow body

Plate 1

Fig. 1. Portion of the mid-gut and Malpighian tube taken from a 24-hour larva. × 340.

Fig. 2. Cross-sections of the head region of a 32-hour larva. × 130.
a. At the level of the cephalic complex.
b. At the level of the ring gland.
c. At the level of the proventriculus.

Fig. 3. Longitudinal section of the posterior part of a 24-hour larva (just after the first moult) showing the high alkaline phosphatase activity of the tracheae. The arrow-head indicates the point of puncture during fixation. × 120.

Fig. 4. Thoracic muscle-fibres of a 32-hour larva showing the concentration of enzyme in the anisotropic disks of the myofibrils. × 340.

Plate 2

Fig. 5. Cross-section of a larva prior to puparium formation showing the contrast between the strong enzyme reaction of the hypodermis and salivary glands, and the negative reaction of the imaginal disks. × 95.

Fig. 6. Cross-section of a 2-hour prepupa at a body level corresponding to that of fig. 5. × 95.

Fig. 7. Sagittal section of an old prepupa just before head eversion. The head region is slightly everted through manipulation during fixation. × 32.

Fig. 8. Longitudinal section of a 12-hour pupa. × 40.

Fig. 9. Cross-section at the level of the wings and legs showing the general increase of alkaline phosphatase after pupation. × 70.

a. 10-hour prepupa.

Plate 3

Fig. 9 *b*. As 9 *a*, 15-hour pupa. × 70.

Fig. 10. Sagittal section of a 32-hour pupa. × 40.

Fig. 11. Longitudinal section of a 72-hour pupa. The intensity of alkaline phosphatase reaction of the nerve cells and proventriculus is much weaker in the actual preparations than can be judged from the photograph. × 40.

Fig. 12. Portion of the abdominal hypodermis taken from a pupa just after head eversion, showing the relative phosphatase activity of the larval and imaginal hypodermal cells. × 340.

Fig. 13. Alkaline phosphatase reaction of the fat-body. × 340.

a. A group of fat cells (in sheet structure) taken from a 10-hour prepupa showing the characteristic reaction of the larval and prepupal fat-body.

Plate 4

Fig. 13 *b*. As 13 *a*. Detached fat-cells taken from a 12-hour pupa showing the tremendous increase of cytoplasmic phosphatase accompanying histolysis. × 340.

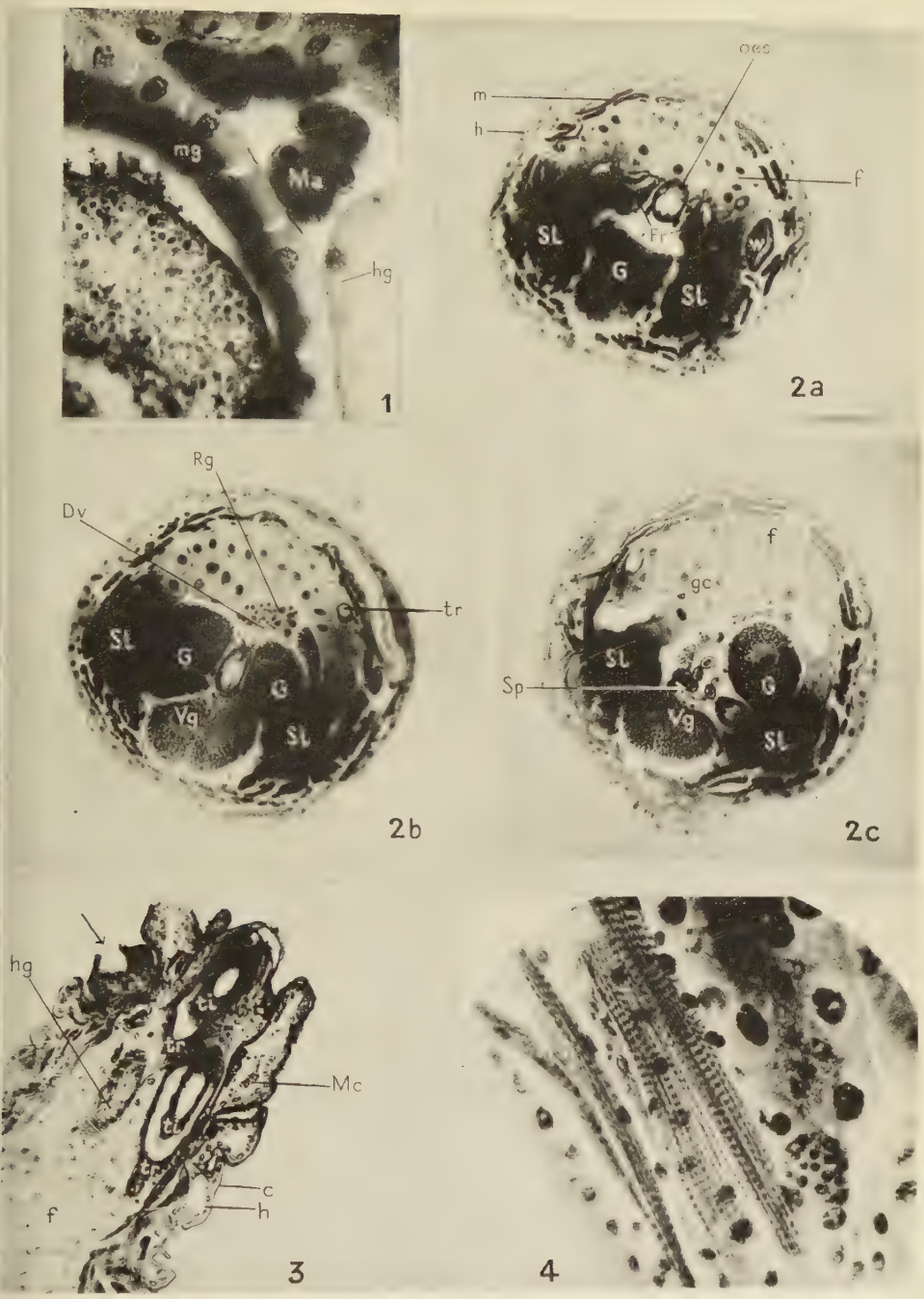
Fig. 14. Section of the ring gland of a 7-hour prepupa showing the difference in phosphatase activity between the corpora cardiaca and corpus allatum. × 340.

Fig. 15. Section through the large pericardial cells, heart, and lymph-glands taken from a pupa just after head eversion. × 100.

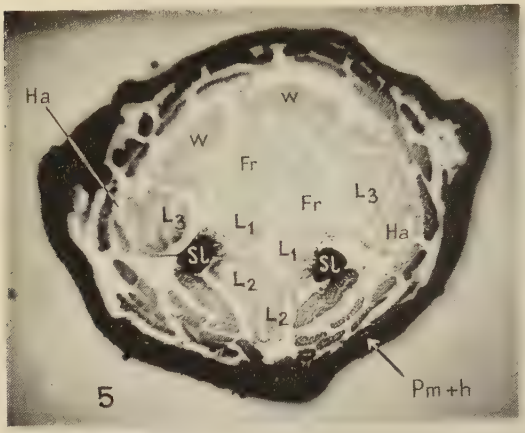
Fig. 16. Portion of the testis from a 32-hour pupa. Note the strong reaction of the sperm heads and the nuclei of the cells of the vas efferens. × 280.

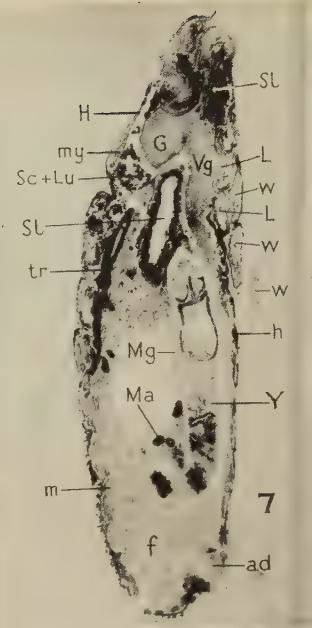
Fig. 17. Section through the ovaries and oviducts of a 48-hour pupa. × 100.

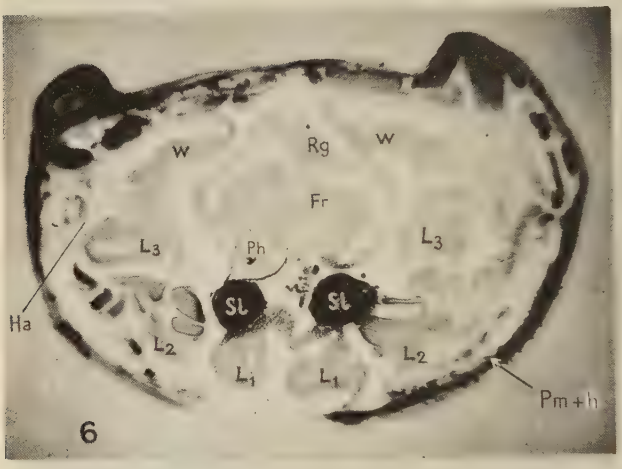
Fig. 18. Section of the ovary taken from an old pupa shortly before emergence. Note the positive reaction of the nuclei of the follicular cells and of the future nurse cells and oocytes. × 280.

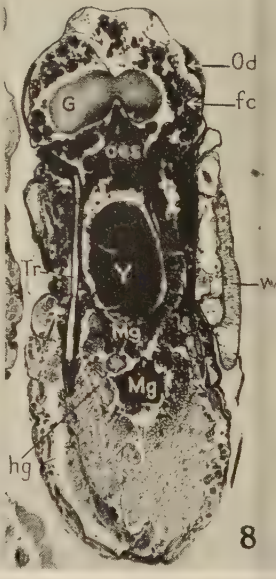


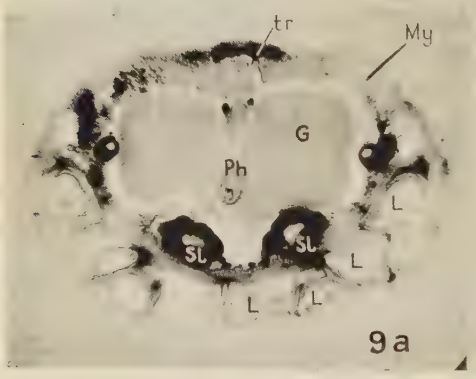
YAO: The localization of Alkaline Phosphatase—PLATE I



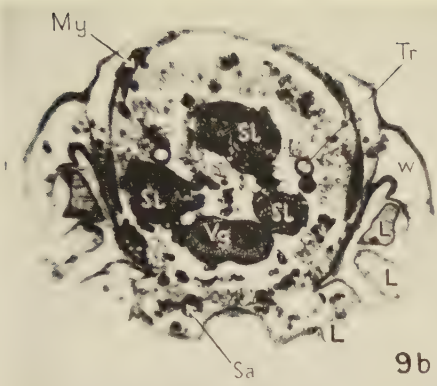








YAO: The localization of Alkaline Phosphatase—PLATE II



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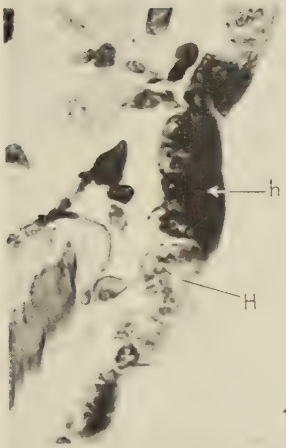

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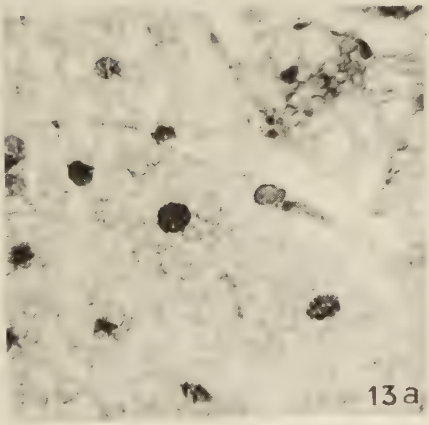

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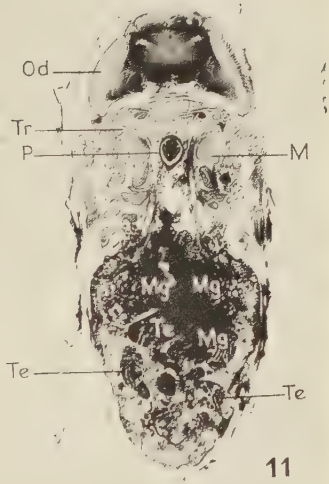

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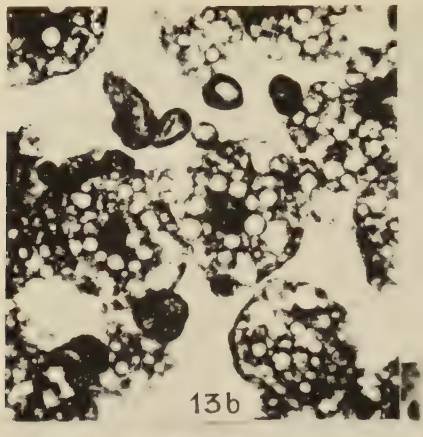
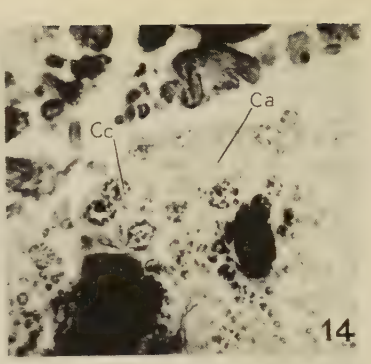
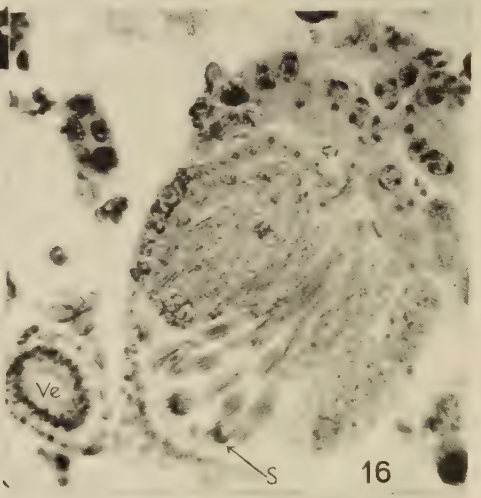
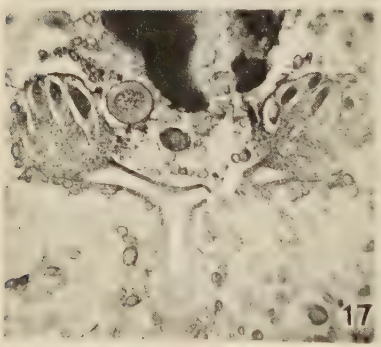
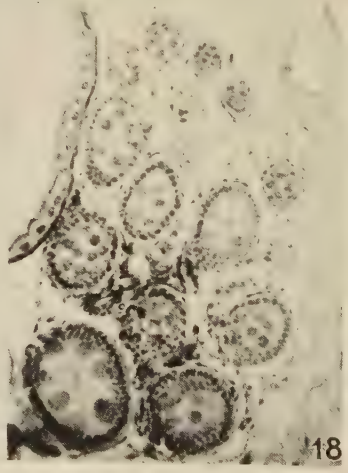
YAO: The localization of Alkaline Phosphatase—PLATE III











YAO: The localization of Alkaline Phosphatase—PLATE IV

The Direct Observation of Cells *in Vitro*

BY

L. G. LAJTHA

(*From the Department of Pathology, Radcliffe Infirmary, Oxford*)

BLOOD or bone-marrow cells in preparations suitable for examination under oil immersion do not usually live for more than a few hours. A technique has been devised in which individual living cells can be observed for 3–5 days by using a specially constructed chamber and an inverted phase-contrast microscope.

The chambers (Fig. 1) are of chromium-plated metal. Two small holes lead into the inside, just wide enough to take a No. 16 needle. Coverslips are

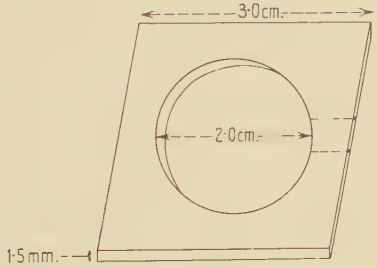


FIG. 1

mounted on both sides of the chambers with 56° C. m.p. paraffin wax. These chambers were designed originally by Dr. A. Q. Wells, Dunn School of Pathology, Oxford. The coverslips and chambers are sterilized separately in an autoclave (the paraffin wax by heating it up to 180° C. for 1 hour), and the coverslips are carefully flamed before mounting them on the chamber.

The cell-suspension is injected through one of the holes into the middle of the chamber, thus avoiding contact with the metallic or paraffin-coated surface, and giving the maximum surface for the gaseous exchange. The amount of fluid injected should be approximately 0·25–0·35 c.c.; the holes should then be sealed with paraffin wax. The height of the chamber allows the use of sufficient culture medium for several days.

The chamber is then placed on the stage of the microscope and after 5–15 minutes the cells sediment and the culture is ready for observation. As the cells sink to the bottom and would be out of reach of high-power lenses an inverted microscope has to be used (Fig. 2), when the cells are easily seen by the $\frac{1}{12}$-inch oil-immersion phase-contrast lens. To project the image into the

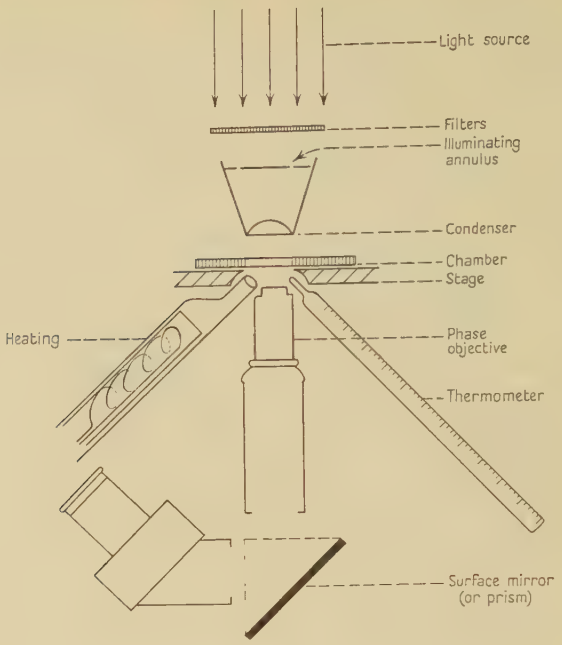


FIG. 2

eyepiece either a prism or a surface mirror is necessary to avoid double reflection phenomena.

The concentration of the cells in the suspension should not succeed 1,000 per cubic millimetre. This dilution gives a distribution in which the individual cells can be followed during their movements, yields a suitably high number of cells for observation, and still maintains a ratio of culture medium to cell volume high enough to keep the cultures alive for several days.

Low-power Phase-contrast Microscopy without a Condenser

By D. A. KEMPSON

(*From the Bureau of Animal Population, University of Oxford*)

SUMMARY

With those low-power objectives in which a phase-plate can be placed near the back focal plane, phase-contrast microscopy can be obtained without the use of any condenser. The illuminating annulus is placed in the conjugate focus of the phase-plate.

ANY microscope may be adapted for phase-contrast microscopy by the method described by Kempson *et al.* (1948) and Baker *et al.* (1949). This method has proved to give excellent results with high powers (2 mm. immersion and 4 mm.). It has now been found possible to attain still further simplification in its application to low powers (e.g. 16 or 18 mm.).

It will be remembered that the condenser forms an image of the annular light-source at a position just below the object, thus producing a secondary light-source, which is then imaged by the objective at a focus behind its rear lens, where the phase-plate is placed. Owing to the rear focal plane of high-power objectives being generally inside the lens-components, and to the limitations of space available inside the mount, it is often necessary to place the phase-plate from one to two focal lengths of the objective behind (above) the rear focal plane. With a low-power objective, however, such as the Leitz No. 3 (18 mm. focal length), it was found possible to place the phase-plate as close to the rear focal plane as 2·7 mm., which is about one-seventh of the focal length of the objective. Consequently, the secondary light-source can be placed some considerable distance away from the object (100 to 200 mm.), much further than with high powers, where it must be only a few millimetres below the object. It naturally follows that under these conditions, the need of even a low-power condenser no longer exists, because the actual light source can replace the secondary source formed by a condenser.

Although theoretically this method is applicable to high powers, there are practical disadvantages that weigh against its adoption. The main objections are (1) the necessity for a very large light-source, and (2) the fact that parts of an ordinary substage will prevent light from the very large annulus required from reaching the object.

Very good phase-contrast was achieved by this method with the objective mentioned. The illuminating annulus must be placed from 100 to 200 mm. from the object (via the mirror). The size of annulus required for any particular

position is easily ascertained by examination of the image formed behind the rear lens (at the position of the phase-plate), with the aid of either a viewing telescope or a low-power objective placed in the draw-tube. The tolerance in the position of the illuminating annulus is due to the fact that precise focus of its image at the phase-plate is not critical, provided that all the direct light passes through the phase-plate annulus.

My thanks are due to Dr. John R. Baker for his constructive criticism and suggestions towards the production of this paper.

REFERENCES

Kempson, D. A., Thomas, O. L., and Baker, J. R., 1948. Quart. J. micr. Sci., **89**, 351.
Baker, J. R., Kempson, D. A., and Brunet, P. C. J., 1949. Ibid., **90**, 323.

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